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Development of tools to detect anthelmintic sensitivity in UK cattle nematodes

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Declaration

The work presented in this thesis is my own work, unless otherwise stated, and has not been submitted for any other degree or professional qualification.

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Claire McArthur Date

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Abstract

Grazing cattle can be infected with a variety of gastrointestinal nematode species. In temperate regions, the most common of these species are considered to be the abomasal parasite, *Ostertagia ostertagi*, and the small intestinal dwelling nematode, *Cooperia oncophora*. Control of these nematodes is largely reliant upon the use of three anthelmintic classes: the benzimidazoles, imidazothiazoles and the macrocyclic lactones (ML). Worldwide, reports of anthelmintic resistance in cattle nematodes have increased in recent years; however, little information is available regarding this issue in farmed cattle in the UK. Knowledge is also lacking regarding anthelmintic usage practices, and whether or not the current methods used to detect anthelmintic resistance in cattle nematodes are robust and accurate.

In this thesis, a number of approaches were taken to investigate these issues. A questionnaire study was conducted to evaluate anthelmintic usage and nematode control practices on a cohort of UK cattle farms. Descriptive analysis of the responses revealed that first season grazing calves were administered with anthelmintics, on average, twice per year (ranging between once and four times per year). It was also found that farmers depended heavily on ML products, with 80% of respondents administering a ML product in the previous 12 months and only 55% of respondents employing a quarantine treatment for cattle brought onto their farm.

Faecal egg count reduction tests (FECRT) were then performed on 20 of the respondents' farms, using injectable ivermectin (IVM). Two types of faecal egg count (FEC) methodology (a double centrifugation salt flotation method sensitive to one egg per gram and a McMaster method sensitive to 50 eggs per gram) were compared to investigate their utility in determining anthelmintic sensitivity of the derived nematode populations. A number of different statistical analyses were also performed to determine the optimum method of analysis for determining anthelmintic efficacy taking into account the effect of using arithmetic or geometric means, sensitivity of the FEC method and the effect of parametric bootstrapping. Of the 20 farms tested, 13 showed indications of inefficacy of IVM. Genus identification analysis of larvae derived from faecal samples obtained from the FECRT cohort indicated a minimum of 95% *Cooperia* spp. larvae in the post-treatment samples. Logistic regression analysis was also used to determine associations between management strategy on beef and dairy cattle enterprises and risk factors for IVM resistance. Farmers with dairy cattle were significantly less likely to use FEC ($P = 0.013$) or isolate new animals

at quarantine (P = 0.013) compared to beef cattle farmers. Farmers who routinely monitored FEC were significantly less likely to use anthelmintics than those who did not monitor FEC (P = 0.042) and farmers who sought advice from their veterinary surgeons were less likely to administer anthelmintics according to the average weight of the herd (P = 0.02).

Nematodes from two of the farms on which IVM resistance was indicated by the field FECRT were then further passaged and both isolates subjected to a controlled efficacy test (CET). Results from the CET confirmed the presence of IVM resistant *C. oncophora* (using two different application methods: injectable and pour-on administration), as well as the presence of moxidectin-resistant *C. oncophora*, the first confirmation in the UK. Analysis of phenotypic parameters was conducted on 679 female *C. oncophora* recovered at necropsy from all treatment groups. For both isolates, nematodes recovered from calves administered with moxidectin (MOX) pour-on anthelmintic were found to be significantly shorter than nematodes surviving IVM administration and nematodes from untreated control calves. Oviposition was also examined; with no eggs *in utero* found in any of the nematodes surviving MOX administration. Differences in the numbers of eggs *in utero* surviving IVM application were observed between the two isolates and also between application methods. Subsequently, analysis of a small section of a glutamate-gated chloride channel (*glc-6*) gene was conducted to investigate the presence of a single nucleotide polymorphism (SNP) in the *glc*-6 gene that had been previously proposed to be associated with ML resistance. This was achieved by comparing sequences derived from male and female *C. oncophora* (from both isolates) obtained from untreated calves with sequences from nematodes that had survived IVM administration. The SNP was not identified in any of the parasites analysed.

Due to time and labour costs for conducting FECRTs for detecting anthelmintic resistance, a migration inhibition test was examined for its utility in assessing IVM sensitivity of third stage larvae (L_3) derived from populations that had been demonstrated to have varying IVM sensitivity *in vivo*. Following optimisation, dose response curves and effective concentration (EC50) estimates were generated for all populations, including single species laboratory isolates of *O. ostertagi* and *C. oncophora* and mixed species isolates derived from the field studies above. The data failed to correlate with the previously obtained *in vivo* anthelmintic sensitivity classification for each isolate. Overall, *O. ostertagi* appeared to be less sensitive to IVM in the LMIT compared to *C. oncophora*, regardless of the ML sensitivity status of the isolate under study. Thus, these experiments indicated that the LMIT may have limited

utility for assessing ML sensitivity of mixed species nematode isolates generated from field samples.

In summary, the work in this thesis has found that UK cattle farmers heavily rely upon ML anthelmintics and there are clear differences in parasite control practices between farmers in the dairy and beef sectors, which may influence the development of anthelmintic resistance. As it is unclear when new classes of anthelmintics will become available for cattle, it is imperative to prolong the effectiveness of the current effective classes and to detect anthelmintic resistance as it emerges. This thesis has explored some currently available tools for the detection of ML resistance, with a view to improving them with appropriate best practice advice to help protect the health and welfare of cattle.

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Chapter 1: General Introduction

1.1 Nematodes that infect cattle and their economic importance

Grazing cattle can be infected with a variety of gastrointestinal nematodes (GIN), with more than 20 trichostrongylid species reported in the UK (Morgan and Soulsby, 1956). The most common genera of GIN cited as infecting cattle globally are *Ostertagia*, *Cooperia*, *Nematodirus*, *Trichostrongylus* and *Haemonchus*, together with the bovine lungworm, *Dictyocaulus viviparus* (Armour, 1989). Within Western Europe, the most prevalent GIN species are the abomasal nematode *Ostertagia ostertagi* and small intestinal-dwelling species, *Cooperia oncophora* (Parkins et al., 1990). The GIN species can be host specific, such as *O. ostertagi*, or infect a variety of ruminants, for example, *C. oncophora* (Stoll, 1936), or *Trichostrongylus axei* (Kates, 1965). The life cycles of all these nematodes are similar, regardless of host specificity or parasite predilection site (Kates, 1965).

1.1.1 General life cycle of trichostrongylid nematodes

Female adult nematodes produce eggs, which are passed out in faeces of infected hosts onto pasture. Trichostrongyle eggs are comprised of three layers: a thick outer layer, which is comprised of remnant material from the lining of the uterus; a chitinous layer, which is strong and responsible for the ability of nematode eggs to survive in challenging environments; and a vitelline layer, containing lipoprotein, believed to have the ability to exclude harmful substances from the inner part of the egg, thus protecting the developing larva (Anya, 1976; Patel, 1997). First stage larvae (L_1) develop within the egg, and are thus protected from environmental

stresses. Under temperate conditions, L_1 develop and hatch within 24 to 48 h, although this can occur more slowly under cooler conditions. The L_1 feed on bacteria and protozoa contained in the faecal pat (Soulsby, 1982) and moult to second stage larvae (L_2), which moult to third stage larvae (L_3), the infective stage. Development from egg to L_3 can occur within 10-14 days of the eggs being passed in faeces in temperate regions (Armour, 1970). The L_3 retain the cuticle of the L_2 as a sheath to provide protection against dehydration and UV radiation and migrate out of the faecal pat (Armour, 1970). Once on pasture, L_3 require a film of water to survive in the environment (Anya, 1976). They migrate up grass blades, where they are available to be ingested by grazing cattle. Following ingestion, L_3 shed the sheath. Exsheathment sites are species-specific, for example, *O. ostertagi* larvae exsheath in the rumen (Frankena, 1987), whereas *C. oncophora* have been shown to exsheath in the abomasum (Frankena, 1987). Once exsheathed, larvae move through the alimentary tract to the predilection site. The predilection site varies between species, with *O. ostertagi*, *H. placei* and *T. axei* (amongst other species) in the abomasum and other species, for example, *C. oncophora*, in the small intestine (Anderson et al., 1965a). Exsheathed *O. ostertagi* L_3 enter gastric glands of the abomasum, where they undergo two moults, before fifth stage larvae (L_5) emerge onto the surface of the mucosa. Alternatively, in the abomasal glands, larvae enter a period of inhibition, known as hypobiosis (Gordon, 1970), where early fourth stage larvae (EL_4) slow development for up to six months before the life cycle continues as described above (Armour, 1970). The exact factors involved in hypobiosis and re-emergence to development are unknown and have been postulated to be due to a combination of environmental and host-mediated factors (Ritchie et al., 1966). Exsheathed *C.*

oncophora L_3 do not enter the crypts of the small intestine, but instead, coil around villi of the small intestine, and are also thought to undergo inhibited development at the EL_4 stage (Armour, 1980). The larvae then moult to fifth-stage larvae (L_5) followed by a final moult to adult nematodes, which mate and the females produce eggs (Soulsby, 1982). Under experimental conditions, the pre-patent period for *O. ostertagi* has been reported to be between 18 and 23 days, with the pre-patent period for *C. oncophora* to be between 11 and 14 days (Wood et al., 1995).

1.1.2 Pathogenesis of GIN in cattle

Parasitic gastroenteritis (PGE) is one of the most important causes of production loss in livestock worldwide, particularly in calves in their first grazing season (Eysker and Ploeger, 2000). This can be the result of clinical symptoms such as diarrhoea, oedema, anaemia and anorexia, or may due to subclinical effects, with reduced daily weight gain and ill-thrift (Eysker and Ploeger, 2000). Infections in older cattle may result in reduced milk yields or fertility losses (Gross et al., 1999). Multi-species infections are common, particularly with *O. ostertagi* and *C. oncophora* and the pathogenicity of *O. ostertagi* is considered a more serious threat to health than the more prolific species, *C. oncophora* (Michel, 1963; Anderson et al., 1965b; Michel, 1968; Armour et al., 1969a, b; Michel, 1969d, c; Michel and Lancaster, 1970; Michel et al., 1970b; Armour, 1974; Michel et al., 1978a; Armour and Duncan, 1987; Armour, 1989; Taylor et al., 1989; Parkins et al., 1990).

1.1.3 Infection with *Ostertagia ostertagi* (Ostertagiosis)

As indicated above, the most economically important GIN in temperate regions is *O. ostertagi*, and is most commonly associated with the symptoms of PGE, although all

GIN species contribute to this syndrome and its associated production losses (Bairden and Armour, 1981). Clinically, ostertagiosis is characterised by diarrhoea, oedema, weight loss, and hypoalbuminaemia, which can result in mortality (Armour, 1970). Epidemiologically, the disease can manifest in one of two ways; this was first described in 1965 after examination of a number of PGE cases in Scottish cattle where *O. ostertagi* was observed at post mortem (Anderson et al., 1965a). First, Type I ostertagiosis is an acute, relatively high morbidity syndrome observed in summer, usually from mid-July onwards, in first season-grazing calves (FSG) as a result of ingestion of substantial numbers of L_3. It is characterised by green, profuse and watery diarrhoea, and is often, but not always, accompanied by high faecal egg counts (for example, in excess of 1,000 eggs per gram of faeces), thirst, weight loss and anorexia (Anderson et al., 1965a). Type II disease occurs in older calves, in the late winter or early spring following their first grazing season. Disease is seen due to the maturation and emergence of hypobiotic larvae from the abomasal gastric gland, with higher levels of hypoalbuminaemia than observed in Type I disease (Armour, 1970). Type II disease typically has a lower prevalence of disease; however, it has been shown that pathophysiological changes can be seen in calves at post mortem without evidence of acute clinical disease occurring (Taylor et al., 1989). Both forms of the disease are associated with weight loss and poor productivity, partially attributable to the damage to the abomasal surface due, described morphologically as a ‘morocco leather” effect due to the distinctive surface nodules induced in response to the larvae and their re-emergence (Ritchie et al., 1966) (Figure 1)

Figure 1: Distinctive nodules observed in *Ostertagia ostertagi* infections (28 days post-infection) associated with larval re-emergence from the gastric glands. (Photo provided courtesy of Dr David Bartley, MRI)

Growth in, and emergence from, the gastric glands results in damage to parietal cells, responsible for the production of hydrochloric acid (Simpson, 2000; Mihi et al., 2013). The resultant reduction in acidity in the abomasum, and associated increase in pH (to above 7), results in a failure to convert pepsinogen to pepsin, and greater numbers of bacteria in the abomasum (Murray and Jennings, 1970). Physical damage, due to worm movement also leads to the abomasal mucosa becoming more permeable, resulting in increased pepsinogen levels in plasma, plasma proteins leaking into the abomasum (leading to a protein-losing gastropathy) and the onset of the clinical symptoms (Armour, 1970).

1.1.4 Infection with *Cooperia* species

In comparison to the pathogenicity observed with ostertagiosis, pathology associated with intestinal species such as *Cooperia*, is considered to be relatively low (Keymer, 1982). *Cooperia* spp. do not encyst in glands but coil around intestinal villi. This can cause widespread villous atrophy in heavy infections and a subsequent loss of brush border enzymes and digestive disturbance (Armour et al., 1987). The

Cooperia spp. which infect cattle include *Cooperia punctata, Cooperia pectinata, Cooperia oncophora* and *Cooperia surnabada,* of which *C. pectinata* is believed to be the most pathogenic as it has been found to enter the small intestinal mucosa (Herlich, 1965b). Cattle infected with *Cooperia* spp. have a catarrhal enteritis and thickening of the gut mucosa (Bailey, 1949). In other studies, although not generally associated with anaemia, a mucosal pallor has also been reported (Herlich, 1965a). Although *C. oncophora* has been presumed to be largely non-pathogenic (Coop et al., 1979), it has been demonstrated that co-infection of this species with *O. ostertagi* can augment poor productivity, inappetence, and has been considered a contributory factor to the effects of PGE in the field (Rose, 1968; Hawkins, 1993; Ploeger and Kloosterman, 1993). Production losses have been recorded in a number of studies when cattle have been infected monospecifically with *Cooperia* spp. For example, a 13.5% reduction in live weight gain was observed over a period of 20 weeks when non-infected calves were compared to calves infected with *C. oncophora*, despite no obvious signs of pathology or clinical symptoms (Coop et al., 1979).

1.1.5 Epidemiology of GIN infection in cattle

Over the course of a typical grazing season, *C. oncophora* has been reported as being responsible for contributing up to 80% of the nematode eggs contaminating pasture and as immunity develops, the majority of adult *C. oncophora* have been shown to be expelled from the host (Albers, 1981). In contrast, *O. ostertagi* has been shown to exhibit a lower peak in egg output and this is usually observed later in the grazing season than *C. oncophora* (Michel et al., 1970b). The longevity of infective L_3 on pasture is a contributing factor to the severity of GIN infections (Michel, 1969a). Larvae can migrate down into soil to avoid adverse environmental conditions and

have been detected at depths of 12.5 cm (Fincher and Stewart, 1979). Whilst a high degree of mortality is thought to occur over winter, given a suitably moist environment, viable L_3 have been recovered from herbage up to two years on rested pastures following grazing by infected calves (Rose, 1961). Studies in Canada demonstrated that *O. ostertagi* and *C. oncophora* L_3 can survive under snow cover in sufficient numbers to cause clinical parasitism in the following grazing season (Smith and Archibald, 1969; Slocombe, 1974). The time taken for infective L_3 to develop from eggs has been shown to be longer over winter months, taking up to 20 weeks to develop, in comparison to approximately 3 weeks observed during the UK grazing season, from April to October (Rose, 1961, 1962). It has been hypothesised that faeces on pasture can act as a reservoir for L_3 and L_3 levels peak when the faeces disintegrate, which is speeded by rainfall and physical disturbance (Michel and Lancaster, 1970). This means that eggs passed out in the previous grazing season can still provide sufficient contamination for susceptible calves to be infected when turned out onto pasture the following season. Such susceptible calves show a peak in faecal egg counts from 3 weeks onwards, providing greater pasture contamination later in the grazing season which is usually associated with the signs of clinical disease in affected animals (Michel, 1963).

Given the perceived differences in pathogenicity between *O. ostertagi* and *C. oncophora*, it would be expected that reports of PGE would identify the prevalence of species present in each case. However, reporting of PGE in surveillance reports rarely identifies the species of GIN involved. As can be seen from the data reported in Figure 2, the majority of cases referred for pathological examination were not

attributed to one species, indicative that a mixed infection may be present. Approximately 10% of referred cases were attributable to infection with *O. ostertagi*, with the highest percentage recorded in 1996 (26% of submitted cases) and the lowest recorded in 2001 (2%).

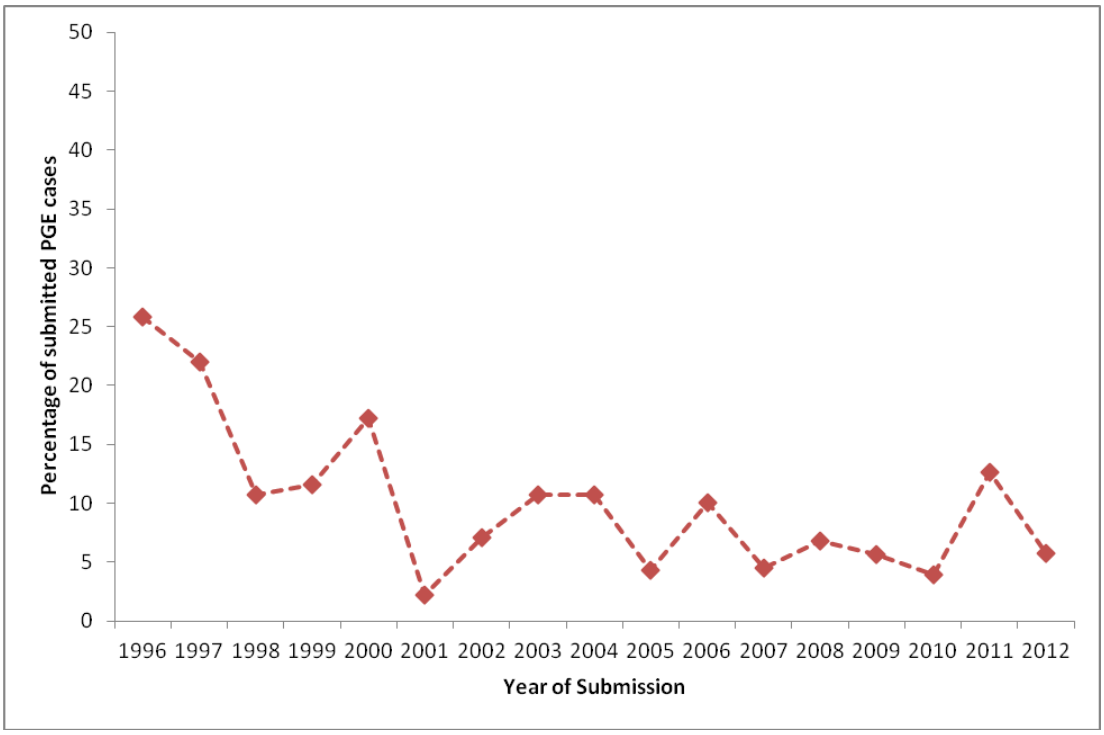

Figure 2: Percentage data of bovine PGE cases attributed specifically to ostertagiosis reported per annum by the Veterinary Investigation Surveillance report data (VIDA; http://www.defra.gov.uk/ahvla-en/category/publications/disease-surv/vida/; accessed 4th May 2014).

1.1.6 Development of immunity

In GIN infections, immunity was shown to be induced in a dose-dependent manner (Armour, 1970). It has been stated that, in the field, natural immunity develops over the course of two grazing seasons. Immunity to *C. oncophora* has been reported to develop within the course of a grazing season, and if exposure to *C. oncophora* is

removed, for example, by housing over the winter months, immunity is quickly re-established upon re-exposure in the next grazing season (Kanobana et al., 2004). Immunity to infection with *O. ostertagi* develops slowly in the field and is generally accepted to develop by the end of the second grazing season (Armour and Duncan, 1987). This delay and the potential for reinfection during this period has been established *O. ostertagi* as the most economically important cattle nematode (Sonstegard and Gasbarre, 2001). Immunity against GIN can manifest in a variety of ways. First, there is a reduction in parasite fecundity, with fewer eggs produced from female worms and passed out to pasture (Michel, 1963). The reduction in eggs produced seems to be a successive downward trend, with each generation of nematodes producing fewer eggs than nematodes in the previous generation (Michel, 1963). This is important epidemiologically as pasture contamination will reduce from a given animal over time following exposure, and is important in clinical assessment, as low faecal egg counts may be observed in animals that still have substantial adult worm burdens (Kanobana et al., 2004). Stunting of nematode length also occurs (Kanobana et al., 2004) as well as direct expulsion of nematodes and reductions in establishment of newly acquired infections (Armour, 1980; Vercruysse and Claerebout, 1997).

1.1.7 Economic effects of GIN infection

It is difficult to ascertain precise figures for economic losses attribute able to GIN infection alone, particularly in the case of sub-clinical disease, where veterinary intervention is often not sought, but losses in production occur (Hawkins, 1993).

Attempts to survey farmers to estimate losses per annum have had limited success; in a US survey, less than half the respondents were willing to place a figure on the cost of parasite infection per head of cattle, with over a third feeling they were unable to accurately judge the extent of infection on their farm (Gasbarre et al., 2001). Those that did, estimated the cost to be $1 - $200 per cow per annum, with the majority of respondents estimating a loss of between $5 and $20 per cow each year (Gasbarre et al., 2001). This figure did not take into account the cost of anthelmintic applications, nor any additional feed rations required to ensure that target weight gain was achieved (Gasbarre et al., 2001). To provide any sort of meaningful figure for the producer, individual farm conditions should be considered, together with factors such as carcass price, reproductive performance and feed conversion rates (Hawkins, 1993). Production losses are not limited to losses observed with sub-optimal slaughter weights. Within the EU, nematode infections have been associated with reduced conception rates in second season grazing calves, longer intervals between calving and conception in adult cattle, both of which can impinge on the profitability of farming (Forbes, 2008). Also, infection with nematodes has been shown to significantly reduce milk yields in adult dairy cattle, by 0.63 kg per day, in comparison to cattle treated with anthelmintics (Gross et al., 1999)

1.2 Treatment of parasitic nematodes in cattle

1.2.1 Anthelmintic products prior to 1960

Prior to the development of broad spectrum anthelmintics in the latter half of the 20th Century, control of GIN relied upon a variety of chemicals such as arsenic derivatives, nicotine sulphate, copper sulphate and carbon tetrachloride; however, issues with efficacy, toxicity and spectrum of activity were inherent with each of these (McKellar and Jackson, 2004). Advances were made with the development of phenothiazine, where doses of 50 to 80 grams were observed to be 100% effective against *Haemonchus* spp., and 80 to 97% effective against *O. ostertagi* (Swanson et al., 1940). However, limited efficacy was observed against *C. oncophora*, and when the dose was increased, toxicity (in the form of blindness and corneal ulceration) was observed, and little reduction in *C. oncophora* faecal egg count was found (Riek, 1951). An organophosphate compound "Neguvon" (Dipterex, Bayer Ltd) was reported to be highly successful in reducing numbers of adult *O. ostertagi* and in controlling an outbreak of ostertagiosis in cattle in Western Australia; however, atropine had to be administered to several animals to combat the symptoms associated with toxicity with this chemical (Banks and Mitton, 1960).

1.2.2 Development of broad spectrum anthelmintic classes

1.2.2.1 Benzimidazoles

The benzimidazoles (BZ) represented the first class of modern broad spectrum anthelmintics and were developed in the 1960s (Brown et al., 1961). These products

showed considerably higher levels of efficacy, together with a wider spectrum of activity and high levels of safety in comparison to the previous anthelmintics (Barragry, 1984). Efficacy is attributed to the selective ability of BZs to bind to nematode β-tubulin, which in turn, results in depolymerisation of microtubules and instability of the nematode cytoskeleton (Lacey, 1988; Lespine et al., 2012). In addition to affecting adult and immature worms by interfering with tubulin binding in intestinal cells and preventing uptake of glucose, BZs possess ovicidal qualities (Martin, 1997; Winterrowd et al., 2003). In the case of thiabendazole (TBZ), inhibition of fumerate reductase activity results in starvation of the nematode, due to the disruption of the parasite metabolic processes (Prichard, 1973). Fenbendazole is the BZ product currently licensed for use in cattle in the UK in the form of oral drenches; an intra-ruminal bolus where an aluminium core is inserted *per os* and corrodes to release discs of anthelmintic over time or as an in-feed mix (NOAH, 2014). Boli provide anthelmintic coverage for periods of up to 140 days and are usually administered early in the year to provide protection throughout the grazing season (Coles, 2002).

1.2.2.2 Imidazothiazoles

Following the success of the BZ anthelmintics, the next group of broad spectrum anthelmintics to be developed was the imidazothiazoles/tetrahydropyrimadine group in the late 1960s (Turton, 1969). Of these compounds, levamisole (LEV) was developed for use in sheep and cattle. An injectable formulation of LEV was shown to exhibit high efficacy against adult *O. ostertagi* and all stages of *C. oncophora*

(Rubin and Hibler, 1968; Turton, 1969). Unlike BZs, LEV is not ovicidal and acts on acetylcholine receptors within the nematode, resulting in a spastic paralysis (Martin and Le Jambre, 1979). It is thought that receptor desensitization may occur when high concentrations of LEV are administered, and so increasing dose rates to achieve greater efficacy was not recommended, particularly as LEV also acts as a nicotinic agonist in mammals and so has a lower therapeutic safety index than BZs (McKellar and Jackson, 2004). LEV is currently only available in the UK for use in cattle as an oral drench, and no products are currently licensed for use in cattle producing milk for human consumption (NOAH, 2014). However, a dermal application method was developed in Australia as a topical pour-on solution, applied directly to the hair (Brooker and Goose, 1975), a method noted for its ease of use and convenience (Bogan and Armour, 1987). The kinetics of LEV absorption through the skin are not well documented, but similar levels of efficacy were observed between oral, injectable and pour-on administration routes previously (Bogan and Armour, 1987). However, lower absorption and efficacy levels than expected have been observed when LEV pour-on preparation was administered in winter months (Forsyth et al., 1983).

1.2.2.3 Macrocyclic lactones

The third broad spectrum anthelmintic class to be developed is the most successful developed to date. Avermectins (such as ivermectin, IVM, and eprinomectin) and milbemycins (such as moxidectin, MOX, doramectin) form the macrocyclic lactone (ML) class, the most used class worldwide, due to high potency, safety index and spectrum of activity against ectoparasites and endoparasites (Campbell, 1981; González Canga et al., 2009). The chemicals, in particular MOX, are highly

lipophilic and have a persistent action (Campbell and Benz, 1984). Both families contain a fused cyclohexene-tetrahydrofuran ring system, a bicyclic 6,6-membered spiroketal, and a cyclohexene ring fused to the 16-membered macrocyclic ring (Zulalian et al., 1994). These compounds exert rapid flaccid paralysis and prevention of pharyngeal pumping within GIN (Wolstenholme and Rogers, 2005), and work conducted on filarial parasites indicates a further role in reducing reproductive activity (Wolstenholme and Rogers, 2005). The paralysis is, in part, thought to be due to the action of MLs on glutamate gated chloride (GluCl) channels (Sutherland and Campbell, 1990; Martin, 1997). MLs irreversibly bind to the GluCl channel, allowing an influx of chloride ions into nematode neuron cells, resulting in hyperpolarisation and paralysis of the parasite (Arena et al., 1991; Cully et al., 1994; Wolstenholme and Rogers, 2005). In addition to the binding of GluCls, MLs have also been shown to act on nematode muscle cells, by enhancing the conductance of the neurotransmitter gamma amino butyric acid (GABA) (Afzal et al., 1994; Brownlee et al., 1997; Feng et al., 2002). IVM has also been shown to be a substrate of P-glycoproteins (P-gp), transmembrane protein pumps in nematodes (Sangster et al, 1994). This is thought to be the main reason why ML compounds are not toxic to most mammals because these pumps are important transmembrane transporters at the blood-brain barrier (Mealey et al., 2001).

1.2.2.3.1 Ivermectin

Avermectins, including IVM, are derived from the fermentation products of *Streptomyces avermitilis*. This bacterium can generate four pairs of closely related compounds; avermectin A_{1a}, A_{1b}, A_{2a}, A_{2b}, B_{1a}, B_{1b}, B_{2a} and B_{2b} (Campbell, 1989). Modification of the structure of avermectin products can affect the potency and

spectrum of efficacy of the resulting derivatives (Egerton et al., 1980): this has led to the further development of derivative B_{1a} and B_{1b} (Burg et al., 1979) as the product IVM. Commercially available IVM contains at least 80% of 22,23-dyhydroavermectin B_{1a} and less than 20% of 22,23-dihydroavermectin B_{1b}). IVM was first registered for use as a broad spectrum anthelmintic in cattle in 1983 and has since become one of the most widely used animal health products worldwide (Prichard and Ranjan, 1993) and considered to be the pinnacle of anthelmintic development in the 20th Century (Geary et al., 2004).

1.2.2.3.2 Moxidectin

Moxidectin is a semi-synthetic derivative milbemycin of another endectocide, nemadectin (Asato and France, 1990). Milbemycin was originally derived from *Streptomyces cyanogriseus* in a sample of red sand in 1983 (Ranjan et al., 1992; Alvinerie et al., 1998) and was registered for use as an anthelmintic for cattle in the early 1990s (Prichard and Ranjan, 1993). MOX is structurally different from IVM in three ways; it lacks a sugar moiety at the C-13 position, has a characteristic methoxime moiety at C-23 and has an unsaturated side chain at C-25 (Zulalian et al., 1994), resulting in a product that is 100 times more lipophilic than IVM. Due to this increased lipophilic nature, MOX has a longer half-life in host fat compared to IVM (Bassissi et al., 2004).

1.2.2.3.3 Doramectin and eprinomectin

In addition to IVM and MOX, eprinomectin was developed and is particularly used in dairy cattle in the UK, due to a zero day milk withdrawal period, a characteristic

unique within this class, due to the portioning between milk and plasma. It is only available as a pour-on formulation (Shoop et al., 1996). Doramectin was developed around the same time as MOX, with the aim to control the dose-limiting *Cooperia* species, as the dose-limiting species for this compound was shown to be the relatively rare, *Nematodirus helvatianus*, in addition to the persistency of this compound in the host (Jones et al., 1993)

1.2.2.3.4 Macrocyclic lactone application methods

After initial development as oral suspensions, an oral boli and subcutaneous injectable formulations, MLs were developed as pour-on applications for cattle. This application method is generally viewed as being the most practicable method of administering anthelmintics due to reduced animal handling time and reduced risk of injury to handlers (Bogan and Armour, 1987; González Canga et al., 2009). The application method has the added benefit of avoiding ML metabolism by the liver and so allowing greater systemic bioavailability (Sallovitz et al., 2002). Pour on anthelmintics are applied along the mid dorsal skin and are absorbed from there. Dose rates are higher than those of injectable and oral formulations to give equivalent levels of efficacy (Leathwick and Miller, 2013); however, a number of concerns have been raised about the use of pour-on applications, especially as a number of factors have been shown to affect efficacy. These include animal breed differences (Sallovitz et al., 2002), hair coat length (Sargison et al., 2009), environmental and husbandry parameters, such as season of treatment (Forsyth et al., 1983); rainfall (Rehbein et al., 1999; Skogerboe et al., 1999) and the action of animals licking each other after treatment (Bousquet-Mélou et al., 2011). It has also been indicated that minor changes to ML disposition (for example, through changing

the application vehicle or excipient used to deliver the anthelmintic) can affect efficacy (Lanusse et al., 1997).

1.2.3 Anthelmintic efficacy at the time of product development

1.2.3.1 Efficacy of benzimidazole products

Thiabendazole efficacy against adult *O. ostertagi* was generally found to be greater than 99% effective in reducing adult worm burden in treated compared to untreated calves (Rubin et al., 1965). However, efficacy of only 55% was reported against *O. ostertagi* L_4 (Rubin et al 1965). Conversely, greater efficacy was observed against *C. oncophora* L_4 (92% reduction in worm burden) compared to adult *C. oncophora* (78% reduction) (Rubin et al., 1965). It was concluded that, compared with the anthelmintic products that predated its release, thiabendazole was more effective at controlling most parasitic nematode species (Hotson, 1963; Rubin et al., 1965). However, a later study observed thiabendazole efficacy was 72.3% against *O. ostertagi* L_4 compared to that of 100% efficacy with fenbendazole (FBZ) (Callinan and Cummins, 1979). Further work looking at the effect of these compounds on inhibited stages of *O. ostertagi* revealed inconsistent efficacies of BZs: these differences have been attributed to small group sizes and different isolates of *O. ostertagi* used in the experimental studies (Duncan et al., 1976, 1977; Williams, 1991) (Duncan et al., 1976, 1977; Duncan et al., 1978). The general consensus regarding efficacy of FBZ against inhibited *O. ostertagi* is further confused by the information provided on product data sheets, with most currently available in the UK stating BZ products are “usually effective” with no further explanation of the level of efficacy (NOAH, 2014).

1.2.3.2 Efficacy of levamisole

Pour-on applied LEV was shown to exert 100% efficacy against adult *O. ostertagi* and *C. oncophora* in one study (Brooker and Goose, 1975); however, later experiments found that efficacy varied between the nematode species, with 92–97% efficacy documented against *O. ostertagi* adults and 99-100% efficacy measured against *C. oncophora* adult worms (Guerrero et al., 1984). No significant reduction in the numbers of *O. ostertagi* or *C. oncophora* L_4 were observed in LEV treated calves compared to untreated control animals (Guerrero et al., 1984). These findings are similar to those documented in other studies (Callinan and Cummins, 1979; Williams, 1991).

1.2.3.3 Efficacy of macrocyclic lactones

1.2.3.3.1 Efficacy of ivermectin

Levels of efficacy observed against *O. ostertagi* and *C. oncophora* exceeded 95% reduction in adult worm burdens in many studies in the development of IVM (Armour et al., 1980; Alva-Valdes et al., 1984; Alva-Valdes et al., 1986). Despite these data, *C. oncophora* is considered the dose-limiting species for ML anthelmintics (Egerton et al., 1979; Egerton et al., 1981) and, generally, higher concentrations of anthelmintic are required to kill > 95% of all nematode stages, in comparison to other species (Ranjan et al., 1992; Scholl et al., 1992). The persistent effect of ML products is shorter against *Cooperia* spp. compared with other genera (Williams et al., 1997). In one study where an IVM pour-on formulation was studied on 15 farms in Australia with no previous use of IVM, the percentage reduction in

faecal egg count was variable against *Cooperia* spp. (Eagleson and Allerton, 1992). Reduction in FEC was greater than 95% on six farms; however, between 30% and 93% reductions were observed on the remaining farms and, in all cases, predominantly *Cooperia* spp., were present in post treatment samples (Eagleson and Allerton, 1992). The survival of *C. oncophora* in nematode populations that were previously not exposed to IVM was also observed in New Zealand, prompting speculation that *Cooperia* spp., may possess mechanisms to better tolerate ML compounds than species such as *O. ostertagi* (Bisset et al., 1990; McKenna, 1995).

1.2.3.2.2. Efficacy of moxidectin

Cooperia spp. are also recognised as the dose-limiting species for MOX (Scholl et al., 1992), as revealed in studies in which MOX, administered at the recommended dose rate, reduced adult male *C. oncophora* by 94%, in comparison to > 99% efficacy achieved against adult *O. ostertagi* (Ranjan et al., 1992). Whereas excellent efficacy (>99%) against *C. oncophora* has been reported in some studies (Williams et al., 1992), a later report found the percentage reduction in FEC 14 days after administration varied between 85 and 94% (Whang et al., 1994). Moxidectin has been shown to have a more persistent effect in the host system than IVM, indicating a use in reducing parasite contamination on pasture (Geurden et al., 2004), It has been suggested that the efficacy of MOX depends on the location of the nematode in the host and that this anthelmintic is not as effective against small intestinal species compared with abomasal species, due to the lower amounts of MOX residues found in small intestinal tissues compared to the abomasum (Eysker and Eilers, 1995).

1.3 Detection and characterisation of anthelmintic resistance

1.3.1 Anthelmintic resistance

Anthelmintic resistance can be defined as a heritable reduction in the sensitivity of an originally susceptible parasite population to the action of an anthelmintic compound (Conder and Campbell, 1995). With continued reliance on anthelmintics for parasite control, the threat of parasites developing resistance increases. As nematode populations are diverse, when individuals able to tolerate a standard drug concentration survive and reproduce, resistance alleles become more prevalent within the population and resistance to the anthelmintic emerges (Prichard, 1990).

1.3.2 *In vivo* methods to detect anthelmintic resistance

There are currently only two *in vivo* methods, which have been recommended for the detection of anthelmintic resistance in cattle nematodes. These are the controlled efficacy test and the faecal egg count reduction test.

1.3.2.1 Controlled Efficacy Test

The controlled efficacy test (CET) is described by the World Association for the Advancement of Veterinary Parasitology (WAAVP) as the 'gold standard' for determining the presence of anthelmintic resistance in helminths of a variety of animal species (Powers et al., 1982; Coles et al., 1992; Wood et al., 1995). It is the most reliable test of efficacy and can be used for virtually all anthelmintics, nematode and host species (Coles et al., 2006). Experimental animals are either naturally infected through grazing pasture, or are experimentally infected with an

inoculum of infective larvae which is deemed sufficient to yield a patent infection, but not so great as to cause clinical disease (Wood et al., 1995). In brief, the techniques assess the efficacy of a compound to remove nematode infections from treated animals compared to untreated infected control animals. If the objective is to examine anthelmintic efficacy based on effects on adult nematodes, animals are treated at approximately 28 days following infection (i.e. once infection is deemed to be patent by the presence of nematode eggs in the host faeces). Animals are grouped, with one group administered anthelmintic at the recommended dose rate, and the remaining group untreated (Powers et al., 1982). The animals are then necropsied seven days later and the surviving adult worms recovered, enumerated and compared between the groups. Originally, the WAAVP considered anthelmintics with an efficacy of greater than 90% to be considered "highly effective" (Powers et al., 1982); however, with the advent of more efficacious products (such as the MLs), this threshold was increased to 98% (Wood et al., 1995). Where anthelmintic efficacy is expected to be greater than 99% in a susceptible nematode population, anthelmintic resistance is confirmed when less than a mean 95% reduction in the adult nematode burden is observed in treated animals in comparison to burdens enumerated in the control group (Coles et al., 2006). As this test requires the use of experimental animals, time, labour, ethical and cost implications need to be considered (Coles et al., 2006).

1.3.2.2 Faecal Egg Count Reduction Test

The faecal egg count reduction test (FECRT) is the most widely used method of assessing anthelmintic efficacy as it can be utilised with naturally- or artificially-infected animals with all classes of anthelmintics (Coles et al, 1992; 2006). The premise is relatively simple, in that faecal samples are taken on the day of anthelmintic administration (i.e. Day 0) and WAAVP guidelines recommend that faecal samples be taken from animals on the day of treatment (Day 0) and at 10 to 17 days following anthelmintic administration. The timing of the second sampling is dependent on the class of anthelmintic administered, with recommendations that samples be taken 10 days after administration with BZ products and between 14 to 17 days following ML administration (Coles et al., 1992). The mean percentage reduction in faecal egg count (FEC) is then calculated, together with 95% confidence intervals. Anthelmintic resistance is reported if the percentage reduction in FEC is less than 95%, and if the lower 95% confidence interval is less than 90%. If only one if the above statements is true, resistance is suspected, rather than confirmed (Coles, et al 1992). There are issues in following these guidelines for assessment of anthelmintic efficacy in cattle in that the guidelines were designed primarily for assessing efficacy in small ruminants and there exists a variety of methods for the detailing various permutations of the reduction calculation (Presidente, 1985; Dash et al., 1988; Coles et al., 1992; Kochapakdee et al., 1995).

Ideally, FECRTs should be conducted in animals excreting a minimum of 150 eggs per gram (EPG) in their faeces; however, as cattle generally have lower FECs than sheep (Coles, et al 2006), this threshold is not always achievable. A more recent

update of the guidelines (Coles et al, 2006) suggested this problem can be circumvented by employing a more sensitive FEC method than that of the standard McMaster method, which has a sensitivity of 50 EPG (Gordon and Whitlock, 1939). A further complication with treating cattle is the variety of anthelmintic application methods available and it has been suggested that when analysing FECRT results, the application method be acknowledged in the determination of resistance status, and a standardised methodology of testing cattle be established to allow a uniform and comparable approach to testing (Sutherland and Leathwick, 2011). It is also difficult to identify surviving eggs to genus and so analysis of L_3 following coproculture is recommended, thus requiring further time and expense as well as expertise identifying the larvae (West et al., 1989). A further limitation of the FECRT is that FECs only provide a measure of adult female nematode fecundity and are unable to determine numbers of juvenile nematodes (Wood et al., 1995). It has been shown that FECs do not correlate well with the actual worm burden in calves (Michel, 1969f; Brunsdon, 1971). It has been suggested that *O. ostertagi* FECs are not correlated with adult worm number, or the probability of developing disease, but instead follow a pattern which depends on the host's immune status, rather than being indicative of total worm burden (Michel, 1969d). A similar finding was found with mixed *O. ostertagi* and *C. oncophora* infections, where calves with FECs of up to 50 EPG were found to have anywhere between 1,000 and 90,000 adult nematodes (Brunsdon, 1971). For these reasons, the WAAVP guidelines for use in cattle are currently undergoing review.

1.3.3 In vitro detection of anthelmintic resistance

As the FECRT and CET have cost and labour implications, *in vitro* methods of detecting and characterising anthelmintic resistance are sought, particularly if they are rapid, sensitive, easily interpretable and capable of detecting resistance earlier than the *in vivo* tests (Shoop, 1993). It has been indicated that the FECRT is only effective in identifying resistance once resistance alleles within a population have reached a prevalence of 25% (Martin et al., 1989). The use of *in vitro* tests to study isolates of anthelmintic resistant ovine nematodes are widely documented and utilise different life cycle stages (Bartley, 2008); however, some tests are limited to a particular developmental stage or anthelmintic compound. The egg hatch test (EHT) is only suitable for screening ovicidal compounds such as BZs and thiabendazole (TBZ) is used in the test. The larval feeding inhibition test (LFIT) (Álvarez-Sánchez et al., 2005), assesses the paralysing effects of LEV and MLs on first stage larvae pharyngeal musculature . The larval development test (Amarante et al., 1984, Demeler et al, 2009) and larval motility test (Demeler et al., 2010b) have been used to characterise anthelmintic sensitivity in cattle nematode populations; however, none of these tests are validated for use with field populations. A test capable of discriminating between sensitive and resistant populations of worms, without being confounded by different species compositions would be of real benefit to diagnostic laboratories in assessing the anthelmintic sensitivity status of field populations.

1.3.3.1 Egg Hatch Test

The EHT is a simple bioassay for assessing BZ resistance in sheep nematode populations (Hall et al., 1978; Le Jambre et al., 1979; Borgsteede et al., 1992; Von Samson-Himmelstjerna et al., 2009). This test has been assessed to a lesser degree

for its utility in assessing anthelmintic sensitivity in cattle nematodes (Demeler et al., 2010b). In brief, freshly extracted nematode eggs are incubated in varying concentrations of TBZ and deionised water for 48 h. As TBZ is ovicidal, eggs from BZ resistant isolates hatch at higher drug concentrations compared to BZ sensitive isolates (Coles and Simpkin, 1977). The comparison of BZ concentrations that prevent 50% of eggs from hatching (ED_{50} estimate) are then used to compare populations. It has been cited that ED50 estimates of >0.1 μg ml^{-1} indicate BZ resistance, but this remains to be validated (Coles et al., 1992) (Coles et al., 2006) and there are no defined ED50 threshold estimates for use of the test with cattle nematodes, particularly in the case of mixed species isolates (Demeler et al., 2012). Furthermore, as the test is only suitable for the detection of BZ resistance, its usefulness is limited in the UK where ML use is far more common (Barton et al., 2006).

1.3.3.2 Larval Migration Inhibition Test

The LMIT, first described in 1979 (Martin and Le Jambre, 1979) is a promising candidate for optimisation with mixed species field isolates of cattle nematodes. It works on the basis of exposing L_3 to a range of ML concentrations and assessing the ability of the larvae to migrate through a mesh of set pore size. The percentage migration is calculated and a dose response curve generated, the results of which are extrapolated to give the concentration of ML required to prevent 50% of the larvae from migrating (Wagland et al., 1992). The LMIT has been optimised for mono-specific isolates of *O. ostertagi* and *C. oncophora*. Optimisation was conducted

using IVM susceptible (IVM-S) and IVM resistant (IVM-R) *C. oncophora* isolates, and with BZ susceptible (BZ-S) and resistant (BZ-R) isolates of *O. ostertagi* as assessed by FECR (Demeler et al, 2010a). As yet, the LMIT has not been optimised for use with mixed nematode species isolated; however, it has been found to be reproducible across different laboratories when used with single species (Demeler et al, 2010b).

1.3.4 Prevalence of anthelmintic resistance

Anthelmintic resistance in small ruminant nematodes has been well documented worldwide, with resistance to BZs, LEV and MLs widespread, and in some places, such as the UK, resistance to all three classes in single populations has been identified (Bartley et al., 2004; Sargison et al., 2005). Although not reported to the same extent as in sheep and goats, anthelmintic resistance in cattle nematodes has been reported, particularly with MLs (Sutherland and Leathwick, 2011). The first case of anthelmintic resistance in cattle nematodes was reported in New Zealand, with *C. oncophora* resistance to oxfendazole (Jackson et al., 1987). The first case of ML resistance was reported in 1995, regarding *C. oncophora* in New Zealand (Vermunt et al., 1995, 1996). In some cases, in particular, in New Zealand and the US, multiple resistance to BZ and ML classes in the same population of *Cooperia* and *Ostertagia* species have been detected (Waghorn et al, 2008; Gasbarre et al, 2009a, 2009b). Researchers in Argentina and Brazil have observed resistance to all three anthelmintic classes in *Ostertagia*, *Cooperia*, *Haemonchus* and *Oesophagostumum* populations (Anziani et al., 2004; Soutello et al., 2007). Within

Europe, several studies have documented ML resistance in *C. oncophora* (Demeler et al., 2009; El-Abdellati et al., 2010), with also a report of LEV resistance in *O. ostertagi* in Belgium (Geerts et al., 1987).

The first case of anthelmintic resistance in UK cattle was reported in 1999, when IVM resistance was confirmed in an isolate of *C. oncophora* (Stafford and Coles, 1999). Since then, there have been few observations of treatment failure and inefficacy following administration of pour-on MLs; however, no large scale surveys have been conducted (Sargison et al, 2009, 2010; Orpin, 2010; Stafford et al, 2010).

1.3.5 The selection of anthelmintic resistant nematodes

Unless an anthelmintic is 100% effective, with every application that is administered, selection for resistant nematodes will occur (Prichard, 1990). The use of anthelmintic products has altered over the past 60 years, in that the majority of anthelmintics were once given to treat the clinical symptoms of parasitism, whereas practices moved towards prophylactic treatment to prevent clinical disease (Michel, 1985). Selection for anthelmintic resistance is an inevitable consequence of anthelmintic use, being a heritable trait which obeys the laws of Mendelian genetics (Sangster, 1999). Anthelmintic resistance generally begins with a small number of nematodes surviving anthelmintic administration, which reproduce and give rise to resistant progeny. Over time, with repeated treatments with the same chemical the number of resistant alleles within the population increases up until the point where treatment failure is observed (Prichard et al., 1980). There are clear indications that the development of anthelmintic resistance is associated with the frequency of

administration (Armour and Bogan, 1982) and with certain management practices, such as under-dosing (Prichard, 1990) or moving cattle to clean grazing (not grazed by cattle in the preceding 12 months) immediately after anthelmintic administration, a process known as "dose and move" (Michel, 1969b; Eysker et al., 1998). By moving cattle to clean grazing immediately after anthelmintic administration, mostly resistant nematodes will contribute to the next generation, instead of being diluted by more susceptible worms already (Prichard, 1990). Currently, parasite management strategies employed by UK cattle farmers are unknown, and so changes to control practices, for example co-grazing or rotational grazing with sheep, are difficult to advise without knowing what is currently happening on farms.

1.3.6 Genetic mechanisms associated with anthelmintic resistance

Benzimidazole resistance in ovine nematodes has been associated with a single nucleotide polymorphism (SNP) at position 200 on the β-tubulin isotype-1 gene in *C. elegans* and *H. contortus* (Kwa et al., 1994). However, this mutation was not consistently observed when a BZ-resistant *C. oncophora* isolate was examined (Winterrowd et al., 2003). A second study also failed to consistently identify a SNP at this site (Njue and Prichard, 2004b). Other studies in ovine and equine nematodes have implicated SNPs for BZ resistance at codons 167, 198 and or 200 in the isotype 1 β-tubulin gene (Kwa et al., 1994).

Whilst LEV is not widely used in cattle, a potential marker has been identified in the down regulation of nicotinic acetylcholine receptor gene, *unc-63a* in two LEV-

resistant isolates of adult *H. contortus* (Sarai et al., 2013). However, this was not observed in a third resistant isolate, indicating resistance may be multigenic in nature (Sarai et al., 2013).

Potential mechanisms behind ML resistance, in particular IVM resistance, have been primarily explored in ovine nematodes. Since the 1990s, work has been conducted in two main areas, focusing on the GluCl channels and ABC transporter P-glycoproteins (Arena et al., 1992; Cully et al., 1994; Pouliot et al., 1997; Xu et al., 1998). Work with anthelmintic resistant cattle nematodes has been focused on GluCl genes, in particular on one gene, AVR14 (Njue et al., 2004; Njue and Prichard, 2004a). Consistent data regarding specific mutations in different isolates has made the discovery of ML associated SNPs a challenge (El-Abdellati et al., 2011). Attempts have been made to standardise the nomenclature used to describe GluCl channels (Beech et al., 2010), but this does not exist for P-gps, and so variously named isoforms are a complicating factor (Kerboeuf et al., 2003). The number of Pgps in parasitic nematodes, included those expressed in non-parasitic stages (such as eggs and L_3), highlights the complexity in defining resistance markers in PgP genes (de Graef et al., 2013b).

1.3.7 Alternative to anthelmintic control of parasites

At the present time, feasible control alternatives to anthelmintics are not available. Work has been conducted on targeted selective treatment (TST) approaches, where treatment is based on live weight gain prediction (Greer et al., 2009) and has been shown to be successful in reducing anthelmintic use in lambs, without affecting

performance (Kenyon et al., 2013). However, a recently published cattle experiment utilising this protocol (Höglund et al., 2013), found that whilst anthelmintic use was reduced by 92%, performance in calves subjected to TST was not as good as in those in which anthelmintics were administered frequently, indicating that further research is required. Other production parameters, such as milk yield and serum pepsinogen levels, have been investigated as diagnostic biomarkers for indicating treatment requirement for *O. ostertagi* infection (Charlier et al., 2005a; Charlier et al., 2005b; Bennema et al., 2009; Bennema et al., 2010; Charlier et al., 2011). A disadvantage of these tests is that they require individual milk or blood samples to be taken and the data generated using these tools cannot be relied upon as a sole measure of parasitism (Charlier et al., 2010b). In addition, there is, as yet, no vaccine available against either *O. ostertagi* or *C. oncophora*; however work is on-going in both parasites (Geldhof et al., 2008; Van Meulder et al., 2013)

Without a short to medium term sustainable alternative to anthelmintics, there is a need to maintain the efficacy of the currently available classes.

1.4 Project aims

The overall aim is to increase knowledge regarding anthelmintic use and parasite management practices and anthelmintic efficacy on UK cattle farms and to evaluate the current tools available for detecting anthelmintic sensitivity. This was achieved by the following:

- Examining anthelmintic treatment strategies implemented on UK farms by questionnaire analysis and identifying risk factors associated with anthelmintic resistance as measured by FECRT.
- Testing efficacy of IVM against nematode populations in FSG calves on a cohort of farms using the FECRT, and identifying the nematode genera present before and after IVM administration.
- Confirming the presence of ML resistance in two *Cooperia* spp. isolates obtained in the field trials above and examining the phenotype and genotype of parasites within these populations.
- Optimising an *in vitro* LMIT for measuring anthelmintic sensitivity in field isolates containing more than one nematode species

By increasing knowledge of the current nematode control practices employed by farmers, and their effect on anthelmintic resistance, combined with the improvement of detection methods, it was anticipated that this research would influence advice to farmers, veterinary surgeons, advisors and the pharmaceutical industry so steps can be taken to ensure the longevity of the current anthelmintic products, whilst maintaining the health and welfare of cattle.

Chapter 2: Parasite control strategies and implementation of “best practice” advice regarding helminth control on UK cattle farms

2.1 Introduction

Anthelmintics are used by farmers worldwide for the preventative control and treatment of gastrointestinal helminth infections in grazing cattle; however, there is little published information available on management practices or measures used to control these pathogens in UK cattle herds (Barton et al., 2006). As a result, there is a lack of knowledge surrounding which anthelmintic classes and application methods are most popular and how these products are utilised on farm. With anthelmintic resistance being reported in cattle nematode populations (Sutherland and Leathwick, 2011), and particularly with the rapid increase in numbers of reports of resistance to the macrocyclic lactone (ML) class over the last decade, it is important to know what farmers are doing to control nematodes on their farms. It is also essential to understand how far their practices adhere to guidelines for limiting the spread of anthelmintic resistance (Coles, 1997). This is particularly pertinent in the cattle sector as no new classes of anthelmintics appear likely to be licensed for use in cattle in the short to medium term (Epe and Kaminsky, 2013). In light of increasing reports of anthelmintic resistance in cattle, an industry-driven initiative has lead to the publication of best practice guidelines, the Control of Parasites Sustainably manual (COWS, EBLEX, 2010), with a view to reducing the risk of anthelmintic resistance developing.

2.1.1 Publication of "Best Practice" Guidelines

2.1.1.1The COWS manual

The COWS manual is a 56-page publication, intended as a reference document for those responsible for prescribing and distributing anthelmintics, such as veterinary surgeons, pharmacists and suitably qualified persons (SQPs), as well as farmers and animal producers. The manual provides an overview of the life cycles and diseases associated with common helminths infecting cattle; the diseases that infection with parasites can cause; the anthelmintics licensed for use in cattle; an overview of anthelmintic resistance in nematodes; laboratory diagnostic techniques (such as faecal egg counts; FEC) and a series of eight guidelines designed to preserve the efficacy and utility of anthelmintics in cattle. The guidelines are comparable to those set down in the Sustainable Control of Parasites in Sheep (SCOPS) guidelines launched in 2003 (Taylor, 2012). A summary of the COWS guidelines is shown in Table 1, adapted from the manual (EBLEX, 2010).

Table 1: Summary of COWS guidelines for sustainable use of anthelmintics on cattle farms (adapted from EBLEX, 2010)

1. Work out a control strategy with veterinary surgeon or advisor
On-going consultations between farmers, veterinary surgeons and farm and animal health advisors to develop a feasible parasite control strategy on-farm, as a culmination of parasitological knowledge and practical understanding of a particular farm (for example, presence of handling facilities and manpower). Must be kept up-to-date based on results from faecal egg count reduction tests (FECRT) conducted on farm.
2. Use effective quarantine strategies

All new stock brought onto farm should be treated with an anthelmintic, ideally with an oral drench formulation of a benzimidazole (BZ; Class I) or an injectable or pour-on application of levamisole (LEV; Class II). Ideally, both products sequentially, but not simultaneously (NB: no time period between administrations is specified). After anthelmintic administration, cattle should be held off pasture for 24-48 hours until nematode eggs have been passed out in faeces and then turned out onto "dirty" pasture contaminated with eggs and larvae deemed to be representative of the farm population. Efficacy of the quarantine treatment should be tested via FEC two weeks post-treatment and if the FEC are in excess of zero eggs per gram (EPG), the animals should be re-treated and retested.

3. Test anthelmintic efficacy on farm

The use of faecal egg FECRT or 'Wormer Tests' (where FECs are only taken after treatment) to determine anthelmintic sensitivity, is encouraged.

4. Administer anthelmintics effectively

Cattle should be dosed at the volume recommended for heaviest in group, based on the weight of two or three of the heaviest animals or to split the group into two subgroups and determine weight of heaviest in group. If the anthelmintic is for quarantine purposes, the full dose rate should be given. Dosing equipment (such as drench guns, syringes and pour-on applicators) needs to be checked "regularly" via the dispensing of two or more doses into a measuring device prior to treatment, using the anthelmintic product.

5. Use anthelmintics only when necessary

The treatment of adult cows is deemed not usually necessary due to development of immunity. Treatment of calves at turn-out should not be necessary as calves should be worm-free, however if pasture contamination is deemed to be high, the use of BZ bolus or a persistent ML (Class III) to prevent disease and further pasture contamination is recommended. Calves should be put onto uncontaminated or low risk pasture at the start of the season, so no need to treat at this time. If pasture is medium or high risk, they should be treated or moved onto silage aftermath after

July. Alternatively, treat at 3, 8 and 13 weeks with ivermectin or 0 and 8 weeks with doramectin or use ruminal boli. FEC are recommended for optimisation of anthelmintic treatment.

6. Select appropriate anthelmintic for the task

Farmers are encouraged to use narrow spectrum anthelmintics where possible, stating LEV may be equally as effective as "a more broader spectrum" ML (*sic*). Inadvertent use of combinations should be avoided and a flukicide used alone if fluke is the target species. The use of a larval culture with FEC is also encouraged, along with rotation of anthelmintics where appropriate (without jeopardising quarantine treatments). The use of products with a persistent action is also encouraged.

7. Adopt strategies to preserve susceptible worms on farm

Cattle should not be treated and moved to new pasture: i.e. "dose and move". However it is noted that delaying the move may be difficult to do on a practical level. It is also noted that there is a lack of evidence on use of targeted selective treatments (TSTs) based on whole herd FEC monitoring.

8. Reduce dependence on anthelmintics

The use of grazing management is recommended, by grazing calves on pasture to allow sufficient exposure in order for immunity to develop, but not so much as to cause disease.

2.1.1.2 Moredun Foundation ACME message

In addition to the COWS manual, there are other advice resources available, such as the Moredun Foundation guidelines (www.moredun.ac.uk). The message in this resource is more succinct than the COWS guidelines, but the focus on the responsible use of anthelmintics remains the same. These guidelines (ACME; named as such after the initials of the guidelines below) were first released for use by sheep and goat farmers; however, the four areas are also applicable to cattle helminth

management and are as follows, with the corresponding COWS guideline number added in brackets:

- Adopt a quarantine strategy (Guideline 2)
- Check efficacy of treatments (Guideline 3)
- Monitor herds to determine when to treat (Guidelines 5 and 8)
- Ensure best practice is followed (Guidelines 4 to 7)

2.1.1.3 Previously conducted questionnaire studies regarding anthelmintic usage

The areas of best practice, as highlighted in the COWS and ACME guidelines, have been used for many years in parasitological studies, primarily conducted in sheep (Taylor, 2012). Questionnaire studies directed at farmers have predominately presented descriptive results, in terms of quoted percentages regarding farmers' general beliefs of their current (and potential future) parasite management systems (Coles, 1997; Sargison and Scott, 2003; Bartley, 2008). More recent studies in sheep, two from Australia (Suter et al., 2004; Sweeny et al., 2012) and one from Great Britain and Ireland (Morgan et al., 2012), detail more advanced analysis of parasite control measures at farm level by incorporation of logistic regression analysis to identify risk factors associated with suspected anthelmintic resistant nematodes on farm and to compare observed signs of disease, and treatment of, liver fluke and lungworm. Suter et al, (2004) generated risk factors for IVM resistance, based on a combination of questionnaire analysis and FECRT data from 120 farms. The results revealed that farmers who applied anthelmintics over the winter months and who had owned farms for longest were more likely to have anthelmintic resistant

nematodes present on farm. Sweeny et al, (2012) focused primarily on the causes of diarrhoea in meat lambs, combined with a geographical and climatic model. In the third study, Morgan et al, (2012), measured the uptake of SCOPS guidelines in on-farm practices in the sheep sector, and found that farmers with less awareness of the SCOPS guidelines were more likely to have poor anthelmintic efficacy compared to those that were aware of SCOPS .

Work conducted in this area with regard to cattle helminth control has been more limited, with only one survey published in the UK in the last 20 years (Barton et al., 2006). A survey conducted in England and Wales by Michel et al (1981), covered the relative "costs" of applying anthelmintics sold in 1978 and farmers decisions behind treatment of cattle, but did not investigate parasite prevalence and was conducted prior to the launch of ML anthelmintics. This research concluded that timing of anthelmintic treatments was not always optimal (for example, when pasture contamination was low) and adult cattle were treated when this may not have been necessary, due to the development of immunity to infection developing over the previous grazing seasons (Michel et al., 1981). A second study, published by Gettinby et al., (1987), focused on the practices of sheep and cattle farmers registered with a veterinary practice. Sixty-eight questionnaires from cattle farmers were received, with 15 'cattle-relevant' questions predominantly exploring usage of anthelmintic class and grazing strategies involving co-grazing with sheep. Farmers were found to treat beef calves on average 1.5 times per annum, with dairy calves treated twice per year on average. Anthelmintic treatments were applied on 84 and 86% of beef and dairy farms respectively, with 84% of dairy farms administering an

anthelmintic treatment to calves at autumn housing (Gettinby et al., 1987). A further descriptive study (Barton et al., 2006), explored the use of anthelmintic classes and application methods on 72 beef farms in South West England. The results revealed 75% of treated first season grazing (FSG) calves with ML product, with ML use rising to 82% with adult cattle. Topical pour-on applications were used by 58% of respondents and 88% of farmers indicating they treated cattle at housing time (Barton et al., 2006). In 2010 a large scale survey of dairy farms in five north-western European countries (including the UK) was conducted, where bulk milk tank *Ostertagia ostertagi* enzyme-linked immunosorbent assay (ELISA) optical density ratios were compared to parasite control practices, such as anthelmintic use and associated management practices such as herd stocking density was predominately descriptive (Bennema et al., 2010). Higher optical density ratios (ODRs) were positively correlated to turn-out earlier in the grazing season and housing later in the grazing seasons and climatic conditions were thought to play a role in differences observed between countries. Further work conducted in Europe has focused on the use of serum pepsinogen levels and grazing management with anthelmintic treatments. A study in the Netherlands found that on 30% of farms, dairy cattle were given preventative anthelmintic treatments, in addition to grazing on aftermath grazing, which is unlikely to provide a substantial parasite challenge, and so anthelmintic treatment may be unnecessary (Borgsteede et al., 1998). Another study in Sweden utilised serum pepsinogen levels to ascertain the subclinical levels of *O. ostertagi* infection in 1% of farms, and FEC levels were below 500 EPG in 97% of cattle tested, to which the authors associated with good pasture management and supplementary feeding (Hoglund et al., 2001).

More detailed research has been conducted in dairy cattle in Costa Rica (Jiménez et al., 2010) and Brazil (Charles and Furlong, 1996). The Brazilian study focused on levels of milk production and farmers decisions regarding anthelmintic administration on a relatively descriptive level, such as 63% of the 89 respondents estimating animal weight to ascertain anthelmintic dosage rates. As with the Michel et al 1981 study, anthelmintic applications were applied indiscriminately across age categories of cattle, resulting in an increase in costs of worm control (Charles and Furlong, 1996). The Costa Rican study focused on parasite prevalence and climatic factors and included logistic regression analysis for determining risk factors for anthelmintic programs, based on the results of 73 farms (Jiménez et al., 2010). A major finding from this study is that although the majority of farmers (90%) claimed to administer anthelmintics at the recommended dose rate, none weighed the animals prior to treatment.

2.1.2 Aims of Chapter

The aim here was to explore anthelmintic usage on cattle farms and which parasite control practices, if any, are implemented in line with advice proposed by initiatives such as COWS and ACME. Data provided by the questionnaire included information on geographical location, farming protocols, enterprise type and parasite management practices which could be examined in light of the farms resistance status. Logistic regression analysis were performed to investigate the association between current best practice guidelines on farming practices and to investigate potential risk factors for anthelmintic resistance on some of these farms.

2.2. Materials and Methods

2.2.1 Questionnaire design and distribution

A questionnaire was devised, based on previously distributed questionnaires for Moredun Foundation sheep farmers by Dr David Bartley. Prior to dissemination, the questionnaire was reviewed by Dr Johannes Charlier (Ghent University, Belgium) and his comments incorporated. The questionnaire was piloted on a group of seven farmers and stockmen, with no changes made after piloting. The finalised questionnaire comprised 44 close-ended questions (Appendix 1) and was designed to cover areas of farm demographics (such as herd size and farm acreage); herd management (for example, time spent grazing and purchase of stock); anthelmintic use (for example, number of anthelmintic treatments per year and dosing regimen); awareness of parasites on farm (including liver fluke and ectoparasites) and sources of advice sought on parasite and nematode control programs. The questionnaire was distributed by post to the 840 Scottish-based members of the Moredun Foundation (http://www.moredun.org/charitable-work/the-moredun-foundation) in July 2010. To increase return rates, the questionnaire was redistributed to the same members the following year (July 2011). Three hundred questionnaires were also distributed to members of the Scottish Beef Cattle Association in November/ December 2010. An information sheet was included in all mailings that listed background information on anthelmintic classes, product brand names and active ingredient in each product and a final flyer was included to offer farmers the opportunity to take part in an IVM faecal egg count reduction test (FECRT).

2.2.2 Data entry and manipulation

Questionnaire responses were entered into an Excel spreadsheet; one row used per questionnaire, one column per question. A final column was used to include any additional information provided by farmers. As some questions produced answers which were under-represented compared to other answers, it was clear that certain questions could be simplified. Each column was subsequently simplified to provide as many binary variables as possible. For example, the question "Do you monitor faecal egg counts?" had three possible responses: "No", "Yes, occasionally" and "Yes, routinely", and was subsequently expanded to form three columns "FEC No" "FEC Occasionally" and "FEC routinely" with Yes/No responses as per Table 2. This was performed for each question, resulting in a data file spanning 300 columns.

Table 2: Example of data simplification: the second column represents the respondent's selected answer with columns three to five displaying the expanded, simplified data. 'NA' indicates an answer was not provided by the respondent

Farm ID	Monitor FEC?	FEC 'No'	FEC 'Occasionally'	FEC 'Routinely'
001	Occasionally	No	Yes	No
002	No	Yes	No	No
003	Routinely	No	No	Yes
004	NA	NA	NA	NA

Following data entry, any missing values were classified as NA (not answered). The data was converted to a comma-separated variable (.csv) file and imported into

RStudio (R version 2.13.1; R studio version 0.97.551) for statistical analysis. Analysis was performed in R Studio using packages 'epicalc' (version 2.15.1.0; Virasakdi, 2012) for deriving odds ratios and 'ggplot2' (version 0.9.3.1; Wickham, 2013) for drawing graphs.

2.2.3 Statistical analysis

2.2.3.1 Selection of variables for analysis

Analysis was directed towards exploring the principles laid down in the COWS guidelines and ACME message. As the principle and intent behind both guidelines is similar, the analysis was designed to encompass five main areas of interest. These are displayed in Table 3 and this combination of COWS and ACME messages shall subsequently be referred to as '*Areas of Best Practice Advice*' (ABPA). In order to address each ABPA and the associated questions for investigation, the questions comprising the questionnaire were allocated to as many ABPA as deemed relevant. For example, questions regarding how FECRTs were performed were allocated to 'checking anthelmintic efficacy' and 'ensuring best practice is followed'. For all ABPA, questions relating to general farm demographics were included, such as farm type and herd size.

Table 3: Areas for statistical analysis: areas of best practice advice (ABPA) displayed on the left column, with associated areas investigated in the right column

Areas of ABPA for analysis	Associated areas of investigation
Adoption of quarantine practices	Are anthelmintics applied for quarantine treatments? Which classes of anthelmintic are used for quarantine? Do farmers isolate new stock post-quarantine treatment? For how long do farmers isolate new stock?
Check anthelmintic efficacy	Do farmers conduct faecal egg count reduction tests? Do farmers feel their anthelmintics are becoming less effective compared to efficacy in previous years?
Monitoring herds to decide treatments	Do farmers conduct faecal egg counts?
Ensuring best practice is followed	Do farmers weigh animals prior to treatment? Do farmers treat their animals on an individual basis ('selectively') or treat as a whole herd or age group? Do farmers calibrate anthelmintic dosing equipment?
Sources of advice sought on parasite control	Where do farmers seek advice about parasite control and anthelmintic use? Which advice sources do they rank as most important?

2.2.3.2 Univariable analysis

Two statistical approaches were used to analyse the data. For the first approach, odds ratios (OR) were generated using univariable logistic regression analysis, referred to subsequently as '*univariable'* analysis. Odds ratios provide an estimate of

the increase (or decrease) in likelihood of an outcome associated with the exposure of a named variable than would be otherwise expected due to chance. Dependent variables were chosen based on their best fit to the ABPA as described in Section 2.2.3.1.

In this analysis, OR were considered significant if the P Wald value (Wald, 1943) was equal to, or less than, 0.05, with 95% confidence intervals (95% CI) outside of 0.95 – 1.04 (i.e. not spanning a value of 1). An OR of 1, or with 95% CI with values between 0.95 – 1.04, was considered to be indicative of a lack of relationship occurring between the variables selected. An OR greater than one, with lower 95% CI greater than 1.04, meant the occurrence of a particular variable increased the likelihood of an outcome occurring by the factor described by the OR. For example, an OR of 12, would indicate the odds of selected outcome occurring are twelve times greater to occur in response to a named variable than would otherwise be expected due to chance. ORs are determined per defined unit, so where numerical categories were involved (for example, number of hectares farmed or number of calves), the OR reflects the likelihood of an outcome occurring for the addition of a single cow or hectare. To address this issue, analysis was conducted using both raw and rounded data values, to ensure the analysis reflected practically relevant figures. Numbers of lambs and ewes farmed (if applicable) were rounded to the nearest 100 animals; pasture size was rounded to the nearest 50 hectares; all cattle groups (adults, stirks and calves) were rounded to the nearest 10 animals, as were the number of rams kept. The length of time that the farm had been owned for was rounded to the nearest 10 years and the percentage of pasture kept as permanent grazing was rounded to the

nearest 10%. Colinearity of variables was considered for each univariable model, by checking all model terms by eye to ensure they were not included – for example, use of LEV anthelmintics and oral forumlations (LEV is only available as an oral anthelmintic).

2.2.3.3 Multivariable univariable analysis

The second approach was to conduct a multivariable univariable logistic regression analysis (Hosmer and Lemeshow, 2000), subsequently referred to here as '*multivariable'* analysis. All output from the univariable analysis with a P value of less than, or equal to, 0.25 was collated and sorted by outcome to be explored. A maximal model was formed to include all explanatory variables, and terms were dropped sequentially by largest P (Wald) value until significance was achieved for all remaining variables ($P < 0.05$). For example, for outcome "CheckGun", relating to whether farmers checked an oral dosing gun prior to use, variables in the univariable analysis such as "BeefAdult" (number of adult beef cattle on farm), "PourOn" (if farmer used pour-ons) and "Bolus" (if farmer used bolus) all had a P value of 0.25 or lower. The maximal model was as shown: CheckGun ~ BeefAdult+PourOn+Bolus and the model run. The term with the highest P (Wald) value ("Bolus") was dropped from the next model run, e.g. CheckGun ~ BeefAdult+PourOn, until all the remaining variables were significant.

2.3. Results

2.3.1 General description of questionnaire data

The response rate was 4.2%, based on the return of 84 questionnaires from 1,980 distributed. Questionnaires were received from 84 respondents, of which 89% (75 farmers) farmed beef cattle, either as a sole enterprise (74%; 62 farmers) or in addition to dairy cattle (15%; 13 farmers). Nine farms (11%) had dairy cattle only. A total of 69% (58 farms) of all respondents also farmed sheep, with the distribution of sheep farmers displayed in Figure 4. Postcodes provided by respondents were entered into publically available, open-source map generation software (www.batchgeo.com) to create the map shown in the left hand pane of Figure 5. The map in the right hand pane of Figure 5 displays the numbers of cattle listed in Scotland in the 2011 Agricultural Census (data obtained through DigiMap EDINA software; http://edina.ac.uk/agcensus/).

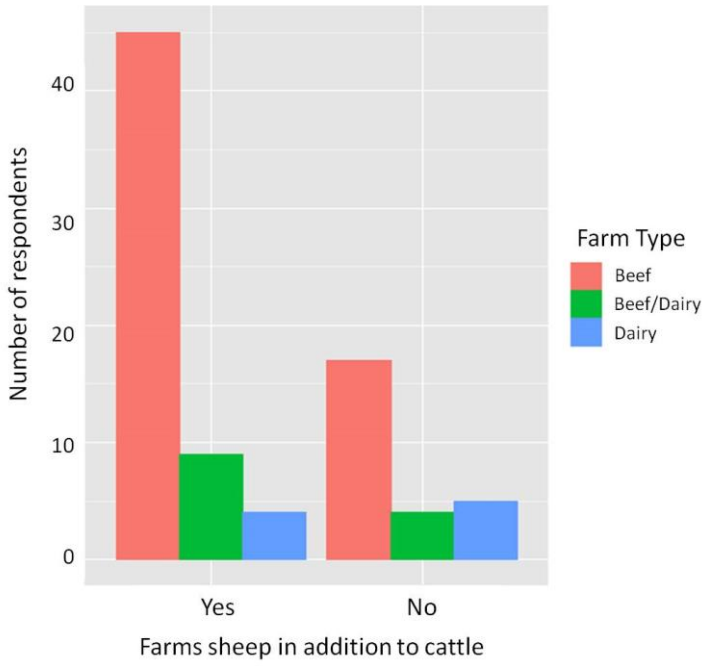

Figure 3: Histogram displaying the distribution of respondents who farm sheep in addition to cattle, across farm types

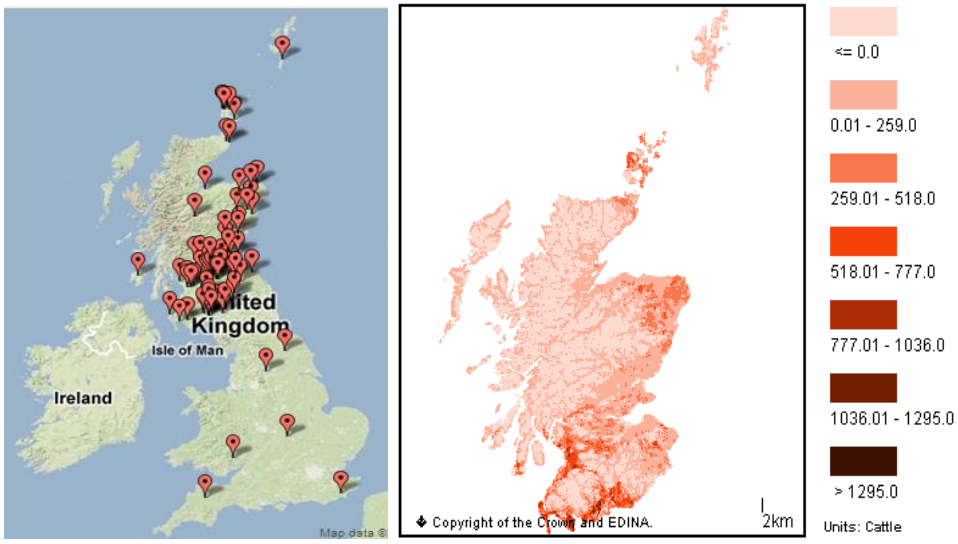

Figure 4: The left-hand pane displays location map of questionnaire respondents, one respondent per individual marker. Right-hand pane displays a map of cattle density, based on total numbers of cattle in Scotland as collated by Agcensus 2011 (Digimap EDINA).

Respondents were predominately located in the central and Southern regions of Scotland, depicting the distribution shown by the cattle density census data (Figure 2.2). Data relating to farm demographics is displayed in Table 4: on average, 52% of pasture owned or rented by the respondents was reported as kept as permanent pasture (i.e. only used for grazing purposes). In general, more adult dairy cattle are kept per farm, compared to adult beef cattle (mean of 151 adult dairy cattle compared to mean of 94 adult beef cattle per farm) however numbers of younger beef cattle (< 24 months; stirks and calves) were higher in the beef than in the dairy sector, with means per farm of 85 and 81 compared to 64 and 56 for stirks and calves, respectively.

Table 4: Farm demographic data, based on replies from the 84 respondents where applicable. For each variable, the mean, median, standard deviation (S.D.) and range are displayed, rounded to the nearest integer.

Variable	Responses (n = 84)	Mean	Median	S.D.	Range
Time land owned or rented (years)	77	48	45	34	4-200
Size of Land owned or rented (Hectares)	82	338	123	915	5-8000
Percentage of land held as permanent pasture	76	52	50	39	0-100
Beef – adult cattle (> 24 months)	70	94	70	120	0-776
Beef – stirks (12 – 24 months)	70	85	45	190	0-1500
Beef – calves (< 12 months)	70	81	62	94	0-532
Dairy – adult cattle (> 24 months)	22	151	132	74	52-360
Dairy – stirks (12 – 24 months)	22	64	58	35	0-150
Dairy – calves (< 12 months)	22	56	50	30	0-130
Sheep - ewes (if farmed)	58	386	140	594	0-3512
Sheep - lambs (if farmed)	58	487	155	751	0-4000
Sheep - rams (if farmed)	58	12	4	20	0-90

Calving occurred year round, but most frequently in March (54 respondents; 66%), April (66; 80%) and May (58; 71%). Similarly, April and May were the months most frequently selected for turnout to grazing, with 78% of animals turned out over these months, across all age groups. Ten respondents (12%) grazed their cattle all year round. Of the remaining farmers, October and November were the most popular months for housing with 62% of adult cattle, 78% of stirks and 74% of calves brought into housing during these months. Sixty-nine percent of respondents co-grazed cattle with sheep (n=55), with the remainder grazing cattle separately (n=25). Sixty-seven respondents (91%) grazed adult cattle for 24 hours per day. Two farmers with beef and dairy cattle grazed adult cattle for 10 or fewer hours per day, and one farmer grazed for 20 hours per day. Three dairy farmers grazed adult cattle for between 12 and 20 hours per day. All stirks and calves were grazed for 24 hours. The majority of adult cattle were grazed at stocking densities of less than three cows per hectare (n=44, 71%), with 35% (n=25) grazed at stocking densities of between three and five cows per hectare. Only two respondents grazed adult cattle at a density of greater than five cows per hectare. A similar pattern was seen with the stocking density of stirks: 47 respondents (70%) grazed less than five stirks per hectare, 19 grazed between five and ten stirks per hectare (28%) and one respondent grazed greater than ten stirks per hectare.

From the analysis (Table 5), farmers who stated that they did not change their grazing pasture over the course of a grazing season (i.e. animals kept on the same pasture from turn-out to housing) were significantly more likely to anthelmintic treat cows and heifers at turn-out (ORs >9.7, 95% CI 1.1-87, P<0.044) and more likely to

farm dairy cattle (OR 3.4, 95% CI 1.2-10, P=0.026). Farmers who stated that they changed their grazing to different pasture during the course of a grazing season were more likely to take advice from magazines (OR 3.5, 95% CI 1.2-10.2, P=0.021) and were less likely to treat heifers at turn-out (OR 0.2, 95%CI 0.1-0.7, P=0.015). Beef cattle farmers were more likely to graze cattle of different age categories together (for example, cows and calves), compared with those who farmed dairy cattle (OR 25.3, 95%CI 4.3-150.5, P<0.001) and were also significantly more likely to seek advice on parasite control from meetings (OR 6.11, 95%CI 1.2-30.1, P=0.026). Seventy-eight farmers stated they treated their cattle with anthelmintics (96% of respondents), with eight farms also using grazing management, and two farms relying solely on the use of grazing management for parasite control, one of which was declared to be organic. On farms where anthelmintics were administered, beef cattle were treated on average 1.8 times per year (SD 0.9, range 0-4) and dairy cattle treated on average twice per year (SD 0.9, range 1-4). Sixty percent of respondents (44 famers) had used ML anthelmintics solely in the previous year, with a further 15 (20%) stating that they had administered ML and BZ class anthelmintics. Eleven farmers (15%) stated that they administered BZ anthelmintics solely and three (4%) stated that they had used levamisole alone.

As shown in Table 5, farmers who administered BZ were significantly more likely to use a bolus application, compared to other methods of application (OR: 3.3, 95% CI 1.2-9.1, P=0.023) and farmers using ML anthelmintics were less likely to take advice from other farmers on parasite control and were also significantly less likely to use oral drenches (ORs 0.2, 95%CIs 0.06-0.08, $P \leq 0.022$).

Table 5: Significant outputs from univariable analysis that related to anthelmintic use and grazing management out with areas of best practice advice

Outcome	Group	Variable	Response (N)	"Yes" response		"No" response	OR (95% CI)	P-value
				N	(%)	N		
Anthelmintic Class	Benzimidazole (BZ)	Bolus	73	13	17.8	60	3.3 (1.2-9.1)	0.023
Anthelmintic Class	Macrocyclic Lactone (ML)	Advice from farmers	76	12	15.8	64	0.2 (0.06-0.8)	0.022
		Use of oral drenches	73	13	17.8	60	0.2 (0.06-0.7)	0.013
Changed grazing	Never	Cows treated at turn-out	54	6	53.7	25	9.7 (1.1-87.0)	0.043
		Heifers treated at turn-out	63	17	27.0	46	8.5 (2.4-30.1)	<0.001
		Farms dairy cattle	80	15	18.8	65	3.4 (1.2-10.0)	0.026
		Advice from magazines	73	17	23.3	56	0.26 (0.1-0.7)	0.008
		Check drench gun	41	12	29.3	29	0.06 (0.01-0.6)	0.013
Changed grazing	During grazing season	Advice from magazines	75	22	29.3	53	3.5 (1.2-10.2)	0.021
		Heifers treated at turn-out	63	4	6.35	59	0.2 (0.1-0.7)	0.015
Different age groups grazed together		Farms beef cattle	74	59	79.7	15	25.3 (4.3-150)	<0.001
		Advice from meetings	70	30	42.9	40	6.11 (1.2-30.1)	0.026
		More than half of grazing pasture mowed per season	72	9	12.5	63	0.21 (0.1-0.8)	0.019
		Cows treated at calving	52	4	7.7	48	0.07 (0.01-0.5)	0.005

2.3.2 Analysis of responses with respect to best practice guidelines

Statistically significant results from all analyses are presented in the following sections, under the corresponding ABPA.

2.3.2.1 Adoption of quarantine practices

Forty-five percent of respondents did not administer a quarantine anthelmintic treatment to animals brought onto farm (33 farms). On the 42 farms for which it was stated that a quarantine anthelmintic treatment was administered, 32 administered a ML class anthelmintic (76%), eight a BZ class anthelmintic (19%) and two administered ML and BZ class anthelmintics (5%). Thirty-three respondents (39%) employed a post-quarantine isolation period, averaging 27 days (range 7-60). In the univariable analysis, farmers were significantly more likely to administer an anthelmintic as a quarantine treatment if new stock were bought at auction (OR 3.7, CI 1.3-10.9, P = 0.018); however, the use of an isolation period of any length was significantly less likely to occur on farms with dairy cattle (OR 0.2, CI 0.1-0.7, P = 0.013, Table 6). In the multivariable analysis, farmers who sought advice on anthelmintic use from pharmaceutical representatives were also significantly more likely to administer an anthelmintic treatment at quarantine (OR 4.1, CI 1.3-13.1, P = 0.017) and isolation after the quarantine treatment was less likely to occur on farms where advice was sought from other farmers (OR 0.25, CI 0.08-0.8, P = 0.021).

Table 6: Summary of univariable analysis of quarantine adoption practices

Outcome	Group	Variable	Response (N)	"Yes" response		"No" response	OR (95% CI)	P-value
				N	(%)	N		
Quarantine Treatment		New stock bought auction	73	18	24.7	55	3.7 (1.3-10.9)	0.018
Isolation Post-Quarantine		Farms dairy cattle	74	4	5.4	70	0.2 (0.06-0.7)	0.013

Table 7: Summary of univariable analysis of use of faecal egg counts (FEC) by farmers

Outcome	Group	Variable	Response (N)	"Yes" response		"No" response	OR (95% CI)	P-value
				N	(%)	N		
Conduction of faecal egg counts (FEC)	No	Farms dairy cattle	79	18	22.8	61	7.1 (1.5-33.4)	0.013
	Occasionally	Farms dairy cattle	79	2	2.5	77	0.2 (0.04-0.7)	0.043
	Regularly	Uses anthelmintics	77	2	2.6	75	0.06 (0.01-0.9)	0.042

2.3.2.2 Check efficacy

Only one farmer stated that he had previously conducted a FECRT on farm. When asked how effective farmers perceived their anthelmintics to be in comparison to previous years, 88% (68 respondents) stated efficacy to be unaltered and 9% (7 respondents) believed efficacy was greater. Only two farmers stated that their anthelmintics were less effective compared to their effect in previous years. No significant correlations were observed in either the univariable or multivariable analyses. Only one farmer stated they had a 'serious' problem with 'worms' on farm, but believed efficacy of anthelmintics used on farm to be more efficacious than in previous years. Of the other respondents, 40% (n=33) stated they did not have any problems with worms, 30% and 23% stated they had 'minor' or 'moderate' problems, respectively (n=24, 19 respectively), and five farmers (6%) stated that they were unsure as to the extent of worm problems on their farm.

2.3.2.3 Monitor herds to decide treatment

Fifty-one respondents (66%) did not monitor FECs at all, with 23 (30%) stating that they undertook FEC analysis on an "occasional" basis. In the univariable analysis (Table 7), dairy cattle farmers were significantly less likely to monitor FECs "occasionally" (OR 0.2, CI 0.04-0.09, P = 0.043) and were significantly more likely not to perform any FECs at all (OR 7.1, CI 1.5-33.4, P = 0.013). Farmers who said they "routinely" monitored FECs were significantly less likely to use anthelmintics (OR 0.06, CI 0-0.9, P = 0.042); however, this was only three respondents. No additional outcomes were found to be significantly associated in the multivariable analysis.

2.3.3.1 Ensure best practice advice is followed

2.3.3.1.1 Effective anthelmintic administration

Farmers were asked how they determined the volume of anthelmintic to administer. Thirty-seven (46%) said that they estimated weights of the animals, with 22 (27%) stating that they administered the dose based on the heaviest animal, 12 (15%) administered on the dose according to weight of individuals and 10 (12%) calculated the dose according to the average weight of the group. In the univariable analysis (Table 8), where farmers stated that they based treatment dose on the weight of the heaviest animal, this was significantly more likely if the farmer also grazed sheep (OR 6.2, CI 1.3-29.0, P =0.021). There was also a significant association between the administration of treatment doses on average weight and the statement that the farmer sought advice on worming practices from their veterinary surgeon (OR 0.13, CI 0.02-0.7, P = 0.02).

Where farmers stated that they used oral drench equipment, the accuracy of the dose gun was significantly less likely to be checked prior to use if the famer stated that he/she estimated the treatment volume to give each animal (OR 0.19, CI 0.04-0.8, P = 0.02). There was also a significant association between the statement that the dose gun was less likely to be checked and where farmers said that they never changed the class of anthelmintic used (OR 0.17, CI 0.04-0.8, P =0.02) or never changed grazing pastures (OR 0.06, CI 0.01-0.6, P = 0.013). In the multivariable analysis, where farmers stated that they checked the dosage gun there was a significant association with a statement that they considered their farm to have had problems with liver fluke (OR 7.11, 95% CI 1.02-50, P = 0.047).

Table 8: Univariable analysis surrounding effective administration of anthelmintics

Outcome	Group	Variable	Response (N)	"Yes" response		"No" response	OR (95% CI)	P-value
				N	(%)	N		
Establishment of treatment volume	Average weight of herd	Advice from vets	74	6	8.1	68	0.1 (0.02-0.7)	0.02
Establishment of treatment volume	Heaviest animal weight	Farms sheep in addition to cattle	77	20	26.0	57	6.2 (1.3-29.2)	0.021
Establishment of treatment volume	Individual weights	Advice from other sources	74	4	5.4	70	4.7 (1.1-20.2)	0.039
		Advice from pharmaceutical reps	74	3	4.1	71	0.2 (0.1-0.8)	0.023
Anthelmintic changed	Annually	Uses an oral drench application	75	7	9.3	68	4.9 (1.4-17.8)	0.016
Dosage Gun checked prior to use		Estimates treatment volume	41	8	19.5	33	0.2 (0.04-0.8)	0.02
		Never changes anthelmintic class	41	7	17.1	34	0.2 (0.04-0.8)	0.022

2.3.3.1.2 Strategic or selective use of anthelmintics

Farmers were asked if they treated the three categories, calves, stirks or cows, on a selective basis (for example, on an individual basis according to need) as opposed to treating the group as a whole in a non-targeted program. Univariable analysis (Table 9), revealed that farmers with dairy cattle were significantly less likely to treat adult cows or calves as whole groups (OR 0.28, CI 0.1-0.8, P = 0.018; OR 0.28, CI 0.1-0.8, P = 0.038, respectively). Farmers who said that they treated all stirks or all calves at the same time on farm were significantly more likely to use pour-on applications (ORs >5.8, CI 1.9-98.7, P < 0.025). The multivariable analysis showed that, in addition to the results found to be significant in the univariable analysis, farmers were also significantly more likely to treat all stirks at once if they also farmed sheep (OR 7.2, 95% CI 1.5-33.8, P = 0.013). Stirk stocking density was found to be associated with the treatment of calves identified by respondents as belonging to a "set worming program" (i.e. a whole group, non-targeted treatment). If the stirk stocking density was less than 5 stirks per hectare, calves were significantly less likely to be treated as a whole group (OR 0.2, CI 0.1-0.8, P = 0.014). However, if stirk stocking density was between 5 and 10 stirks per hectare, calves were significantly more likely to be treated as a whole group (OR 3.5, CI 1.1-11.2, P = 0.033).

Table 9: Univariable analysis regarding selective and strategic treatment of cattle

Outcome	Group	Variable	Response (N)	"Yes" response		"No" response	OR (95% CI)	P-value
				N	(%)	N		
Non-selective Treatment	Adults	Farms dairy cattle	77	7	9.1	70	0.3 (0.1-0.8)	0.018
Non-selective Treatment	Calves	Advice from vets	76	59	77.6	17	7.9 (1.5-40.6)	0.014
		Pour-on application	77	59	76.6	18	5.9 (1.3-27.5)	0.024
		Also farms sheep	78	48	61.5	30	4.0 (1.2-13.3)	0.024
		Farms dairy cattle	78	14	18.0	64	0.28 (0.1-0.8)	0.038
Non-selective Treatment	Stirks	Pour-on application	76	58	76.3	18	17.4 (3.1-98.7)	0.001
		Advice from meetings	75	23	30.7	52	0.3 (0.09-0.9)	0.04
Anthelmintic treatment of calves	Set programs	Stirk stocking (5-10 per hectare)	60	10	16.7	50	3.5 (1.1-11.2)	0.033
		Stirk stocking (<5 per hectare)	60	10	16.7	50	0.2 (0.1-0.8)	0.014

Anthelmintic treatment of calves	At turn-out	Advice from meetings	71	5	7.0	66	0.2 (0.1-0.6)	0.006
Anthelmintic treatment of calves	At weaning	Advice from magazines	71	5	7.0	66	0.2 (0.1-0.7)	0.009
Anthelmintic treatment of cows	At calving	Farms dairy cattle	56	5	8.9	51	8.3 (1.7-42.1)	0.01
		Farms beef cattle	56	5	8.9	51	0.1 (0.02-0.7)	0.02
		Stirk stocking (<5 per hectare)	48	2	4.2	46	0.1 (0.02-0.7)	0.017
		Adult stocking (<3 per hectare)	50	1	2.0	49	0.1 (0.01-0.6)	0.016
Anthelmintic treatment of heifers	At signs of disease	Pour-on application	63	3	4.8	60	0.1 (0.01-0.9)	0.036
Anthelmintic treatment of heifers	At turn-out	Bolus	63	13	20.6	50	6.0 (1.9-18.8)	0.002
		Advice from magazines	61	10	16.4	51	0.3 (0.1-0.9)	0.036

2.3.3.1.3 Preservation of anthelmintic susceptible helminths

Farmers who stated that they treated animals before moving them onto different pasture ("dose and move") were significantly more likely not to give any supplementary feeding such as silage (OR 5, CI 1.2-20.3, P=0.024, Table 10). Farmers were significantly more likely to graze mixed-age groups of cattle if they farmed beef cattle (OR 25.29, CI 4.3-150.2, P<0.001) and if they used farmers meetings as a source of advice for parasite control (OR 6.1, CI 1.2-30.1, P=0.026). Only three respondents (3.6%) did not use anthelmintics on their farm, all of whom farmed beef cattle with one classed as organic. The remaining two were not organic, but the farmers stated they used grazing management in place of anthelmintic treatments for parasite control. On farms where anthelmintics were not used, the mean herd sizes were 92, 67 and 77 for adult cattle, stirks and calves respectively. These figures correlate well with the mean herd sizes reported by all respondents for beef cattle (as displayed Table 4) with mean herd sizes of 94, 85 and 81 for adult cattle, stirks and calves respectively. A further six farmers stated that they used grazing management in addition to anthelmintics for helminth control.

2.3.4 Sources of advice on helminth control practices

From the responses, veterinary surgeons were cited as the most selected source of advice for anthelmintic use and helminth control (71 respondents, 89%), followed by magazine articles (48; 60%), pharmaceutical company representatives (42; 53%), farmers' meetings (36; 45%), advertisements (30; 38%), other farmers (23; 29%) and sources other than those mentioned above (10; 13%). When asked to rank the most important sources of advice, veterinary surgeons were selected by 48 of the respondents (76%). Farmers who stated that they sought advice from magazines (i.e.

farming press and related publications) were significantly more likely to state that they had problems with mites and lice (OR 4.2, 95% CI 1.4-12.1, P = 0.008) and were also more likely to farm sheep as well as cattle (OR 2.9, CI 1.1-7.9, P = 0.037). As seen in Table 11, farmers who ranked ‘meetings’ as their most important source of information regarding control practices were significantly more likely to be unsure if worms were considered a problem on their farm (OR 38, CI 2.6-548, P = 0.037).

Table 10: Univariable analysis regarding preservation of susceptible worms on pasture

Outcome	Group	Variable	Response (N)	"Yes" response		"No" response	OR (95% CI)	P-value
				N	(%)	N		
Dose and move		No supplementary feeding	76	9	11.8	67	5 (1.2-20.3)	0.024

Table 11: Univariable analysis surrounding sources of advice on anthelmintics used by farmers

Outcome	Group	Variable	Response (N)	"Yes" response		"No" response	OR (95% CI)	P-value
				N	(%)	N		
Advice Source	Magazines	Problems with mites and lice	77	24	31.2	53	4.2 (1.4-12.1)	0.008
Advice Source	Magazines	Farms sheep in addition to cattle	78	38	48.7	40	2.91 (1.1-7.9)	0.037
Advice Source	Meetings	Farms dairy cattle	78	4	5.1	74	0.18 (0.1-0.6)	0.006
Advice from source ranked most important	Meetings	Unsure of worm problem	63	2	3.2	61	38 (2.7-546.7)	0.007

2.4. Discussion

The main objective here was to gain information about current helminth control practices on cattle farms and to compare how the cited practices align with those written in the publically available best practice guidelines. The results indicated that farmers are not always following the best practice advice and the barriers to this must be ascertained. The response rate here was low. This has been reported to be common in studies such as this (Coles, 1997). However, in other studies, notably in Belgium, response rates of 61% and greater than 90% have been recorded (Charlier et al., 2010; Claerebout et al., 2000). One issue with obtaining such a low response rate, is the increased potential for bias, for example, there is no way to determine if these respondents were particularly interested or well-informed in regard to the subject prior to the survey. As a result, the practices of these respondents may not be indicative of the general population of cattle farmers in the UK. A personal interview approach has been used in surveys conducted with sheep farmers in Northern Ireland (McMahon et al., 2012) in an attempt to increase response rates. Given that the actual number of interviews conducted was only 81, the benefit of this approach may be limited. Other studies have targeted farmers via telephone questionnaires (Morgan et al., 2012) and via veterinary practices (having been identified as being more likely to respond) (Stafford and Coles, 1999); however, both approaches introduce their own bias. For example, in a study conducted on farms served by a veterinary practice (Sargison et al, 2003), farmers were surveyed before a meeting held by the practice and participants were noted to be, on the whole, owners of large well-established farms with high levels of veterinary care and higher than average output compared with other sheep farms. Bias may occur when recording

responses from these as farmers who attend meetings may be the most interested in keeping up-to-date, so may be better informed than the population as a whole. Alternatively, farmers may also be more likely to take part if they suspect they may have a problem and so bias may be implicated in that instance (Thrusfield, 2007). Even when farmers have previously expressed a willingness to participate in questionnaire studies, return rates can still be lower than expected (Stafford and Coles, 1999), for example, a return rate of 80% was observed with the study by Barton et al., 2006, despite previous assertions that farmers were willing to participate (Barton et al., 2006). Other, more qualitative methods may also be employed to gain insight into the practices and opinions of farmers, such as the use of focus groups or a more conversational interview approach, where the questions are open-ended, leading to a discussion between the interviewer and the interviewee (C. Jack, Moredun, Personal communication).

The issue of low response rates may also be, in part, due to the current perceptions of the importance of anthelmintic resistance in the cattle sector. Due to the relatively few cases of anthelmintic resistance reported in cattle nematodes in the UK (Stafford and Coles, 1999; Sargison et al., 2009; Sargison et al., 2010; McArthur et al., 2011; Bartley et al., 2012), it could be conjectured that farmers may not be as aware of this issue as sheep farmers. The findings here revealed that 70% of respondents believed they had no problems or minor problems with parasitic nematodes on their farm. If more cattle farmers recognised the potential threat of anthelmintic resistance and its effects on parasite control, then the response rate may have been higher. Oppenheim (1992) has suggested a number of ways of increasing questionnaire response rates,

including sending reminder notices and the importance of an introductory letter to highlight reasons for the survey. Both of these approaches were tried here, and the questionnaire designed to be as easy to complete as possible (through the use of close-ended questions), but the response rate remained low. The same author (Oppenheim, 1992) also suggested incentives to increase response rates, and in the instance of this study, farmers were offered the opportunity to conduct a FECRT inclusive of all required materials (including anthelmintic). Similarly, a previous study surveying the nematode control practices of UK sheep farmers (Coles, 1997) used a prize draw with the chance to win one of five £25 vouchers to encourage participation and had a return rate of 22.8% (684 questionnaires). Although the questionnaire here was kept as short as possible to aid comprehension and willingness to participate, it may have been informative to include a question pertaining to clinical signs of parasite infection (such as poor weight gain or diarrhoea). In a survey of sheep famers in Australia, a photographic display of the "dag" reference chart for breech soiling (scored from 1 "no evidence of soiling" to 5 "heavy soiling") has been used in an attempt to assess this (Sweeny et al., 2012); however it may require a caveat as other infections can cause diarrhoea, and that diarrhoea may not necessarily occur during parasite infection. Due to the low response rate here, care must be taken in extrapolation of the results, as they do not represent an expansive survey, but the information garnered does add to the information previously published in smaller surveys (Barton et al., 2006). In addition, the location of the farms from where the data was collated follow areas of high cattle density, so even with small survey size, respondent location appears to be representative of the cattle density in Scotland.

Due to the lack of published data regarding cattle anthelmintic treatments and management regimes, it is difficult to make comparisons to other studies. In terms of general practices such as turn-out and housing, these reflect similar findings previously published in Northern Europe. In the UK, calves , particularly beef calves were turned out in April or May with their dams, grazed all summer and housed in October or November in preparation for winter (Michel et al., 1981; Gettinby et al., 1987). In Belgian studies, (Charlier et al., 2005a; Bennema et al., 2010; Charlier et al., 2010a), which focused on the dairy sector generally, cattle are turned out in spring and housed in late autumn or winter and grazed extensively, with the majority grazed for 24 hours a day during the summer season. With regards to levels of treatment and classes of anthelmintics applied, similar findings (averaging one to two treatments per year, predominantly with ML products) were found by Barton et al (2006), where 75% of respondents administered some form of ML anthelmintic to beef calves, increasing to 82% for adult cattle. In contrast to the current survey, only 29% of respondents received parasite control advice from their veterinary surgeons. An earlier study (Gettinby et al., 1987) found that although farmers that employed grazing management practices were more likely to treat more frequently, anthelmintic treatment of calves was at similar levels to those reported here (average 1.5 and 2 times per year, beef and dairy, respectively).

It is clear from the results that there is confusion regarding appropriate quarantine anthelmintic treatments for cattle. The fact that farmers who bought new stock at auction were found to be significantly more likely to administer a quarantine anthelmintic is indicative of an awareness of biosecurity; however, it is less

encouraging when the selected anthelmintic is examined. The current best practice advice is to give a BZ treatment, or a LEV treatment or both treatments sequentially (EBLEX, 2010). This is because anthelmintic resistance in cattle nematodes has only been reported in MLs to date. The suggested use of a BZ administration in addition to LEV is that LEV is not effective against inhibited *Ostertagia* spp. (Grimshaw et al., 1996). The results here showed that only 40 respondents gave any anthelmintic at quarantine and of those that did, only six administered a BZ class anthelmintic and none administered LEV. Two farmers administered a BZ class treatment as well as a ML treatment, but it is unknown how close together administrations were given. This confusion is not limited to the cattle sector, as the study by Morgan et al (2012) revealed that 86% of sheep farmer respondents believed they were administering the correct quarantine anthelmintics to new stock, whereas only 3% were administering LEV and MOX (the best practice advice for sheep at the time, SCOPS 3rd edition;(Abbott et al., 2009)). Sargison and Scott (2003) also found differences in quarantine procedures compared to best practice advice, as 17% of respondents from their survey administered IVM or doramectin (other ML class anthelmintics) and not MOX and LEV. The average length of isolation periods 27 days compared to the 24-48 hours stipulated under best practice advice (EBLEX, 2010). This may be attributed to farmers isolating for other diseases, such as Bovine Viral Diarrhoea virus infection, acknowledged to be a concern when purchasing new cattle (Brennan and Christley, 2012). If farmers are already employing a lengthy quarantine period, this may be the ideal opportunity to promote use of an effective anthelmintic quarantine treatment, potentially combined

with a FECRT to examine efficacy of treatment, as part of a cohesive herd health approach.

Clear trends have been shown from the survey results, particularly with the differences between beef and dairy farmers in relation to best practice advice. This could be attributed to the fact that there is a positive, albeit not statistically significant, association between beef cattle farmers and farmers that also raise sheep in addition to cattle. This might have an indirect influence on treatment of cattle parasites, as the SCOPS guidelines have been published since 2003, and so the message of sustainable use of anthelmintics has been promoted in the sheep sector for much longer than in the cattle sector, particularly as anthelmintic resistance in UK was not published until 1999 (Stafford and Coles, 1999).

Monitoring FECs was found to be significantly less likely to occur on farms with dairy cattle. This finding is in agreement with a study conducted in Costa Rica, where no FEC were conducted prior to treatment and decisions were based on subjective appearance of clinical signs (including "sad animals" or diarrhoea) or change of season (such as from low to high rainfall period) (Jiménez et al., 2010). Only one farmer had previously conducted a faecal egg count reduction test, and provided additional information revealing that it was conducted using LEV and provided 100% efficacy. From the rest of his questionnaire completed, it was apparent that this farmer was only using ML products on farm, so the results of the LEV FECRT may be limited in their application at the present time. This result is not unique to the cattle sector, as it has been previously asserted that few sheep farmers currently assess anthelmintic efficacy in their flocks, and are subsequently

unaware of the potential extent or the true resistance status of their farm (Morgan et al., 2012). This may be linked to farmers' belief that their anthelmintics are still as effective compared to previous years, with only two believing they were less effective. However, it has also been proposed that farmers may hold unrealistic expectations as to anthelmintic effectiveness, particularly if they are being used to improve the condition of animals who may have underlying or unknown ailments (Riffkin et al., 1984).

The use of targeted anthelmintic treatments in cattle has been proposed since the 1980's when a survey by Michel et al, (1981) used the number of anthelmintics sold in 1978 to produce an analysis of administrations to different cattle age groups and equated that only 20% of this cost was spent on "potentially useful" treatments. It was stated that the remaining 80% spent on anthelmintics that year was used in treatments of "doubtful or marginal" value, or "outright wasted" (Michel et al., 1981). The use of FEC to ascertain levels of pasture contamination and so determine potential benefits of deciding when best to administer anthelmintic treatment should be continued to be disseminated to farmers. This view is shared by researchers in The Netherlands who predominantly investigated the use of anthelmintic treatments in the control of lungworm infection in calves. Through the use of 'inappropriate' treatments, such as the use of a non-persistent anthelmintic at turn-out, and the use of persistent anthelmintics during winter housing, it was postulated that farmers may be in danger of 'overprotecting' their herds and better decision making processes were required (Borgsteede et al., 1998). Overprotection was considered to be present when an anthelmintic treatment program was still

providing protection against re-infection when the grazing season ended and subsequently followed by an anthelmintic treatment at housing (Borgsteede et al., 1998). Use of anthelmintic treatments in this way provides strong selection for anthelmintic resistance. The degree of selection pressure is dependent upon the intensity of selection pressure through the frequency of treatments, the dose rate applied, the proportion of larvae left untreated and the contribution of larvae which have survived anthelmintic treatment, either through resistance or surviving a sub-optimal application (Prichard et al., 1980).

Here, farmers who stated that they treat their animals to the average weight of the herd were found to be significantly less likely to seek advice on worming from their veterinary surgeons. A study in Costa Rican dairy cattle showed that although 89.8% of the farmers surveyed stated they administered anthelmintics at the recommended dose rate, none of the farmers weighed animals prior to treatment (Jiménez et al., 2010). Barton et al (2006) reported cattle were weighed on only 36% of the farms surveyed in their study, whereas here, 42% of respondents administered doses to either individual or heaviest weights in their herds. It has previously been asserted that the determination of accurate dose rates by weighing animals, or using weigh tapes to estimate weight, is of utmost importance for anthelmintic efficacy (Cabaret and Berrag, 2004). The finding that farmers who treated to the heaviest weight in the herd were significantly more likely to farm sheep is encouraging, as it is a recommendation in the SCOPS guidelines and demonstrates an important flow of information from the sheep to cattle sector. Whereas dosing a group of animals to the heaviest weight may be considered wasteful, as some animals will receive a

larger volume of anthelmintic than is strictly required, the alternative of potentially underdosing animals by basing treatment volumes on estimated body weight provides stronger selection for anthelmintic resistance (Smith et al., 1999).

Farmers who stated that they did not treat calves on a selective basis were significantly more likely to seek worm control advice from their veterinary surgeons. This may be a factor of farmers wishing to maintain, or increase, productivity with the belief that the absence of parasites will increase growth rates and result in higher levels of productivity (Michel, 1985). Those farmers who sought veterinary advice were significantly more likely to use a pour-on product, which may be due to the convenience of this application method (Bogan and Armour, 1987). The use of a whole group, non-targeted treatment program to treat stirks kept at medium stocking density (5-10 per hectare) could also be indicative of convenience to the farmer. As with 'overprotection', the reduction of the population of untreated larvae ("in refugia") will increase the selection pressure on pasture, as there will be fewer untreated larvae developing on pasture to dilute larvae surviving anthelmintic treatment (Van Wyk, 2001).

A difference was observed between dairy and beef cattle farmers with regards to anthelmintic treatment at calving with dairy cattle significantly more likely to be treated then and is probably due to the perception that anthelmintic treatment at calving increasing milk yields (Bisset et al., 1987). Previous studies found no effect on milk yield with the application of BZ or LEV products at calving time (Michel et al., 1982); however, a review of studies conducted during dry season or mid-lactation, showed varying levels of increased milk production in various countries

(Gross et al., 1999). As adult cattle are known to have low FEC and are likely to have developed a substantial degree of immunity following the previous grazing seasons, anthelmintic treatment for parasitic disease is not always necessary (Michel, 1985). In addition, refugia on pasture from not treating older calves and adult cattle has been considered an important reason why anthelmintic resistance is not more widely found in cattle (Coles, 2002). However, with the development of eprinomectin (ML), milk yields were found to be significantly increased following administration (Reist et al., 2011). Eprinomectin was developed as the first ML product with a zero milk withdrawal period, and as such, could be used in lactating dairy cattle (Shoop et al., 1996a; Shoop et al., 1996b) and has also been shown to be associated with increased weight gain in treated cattle (Kunkle et al., 2013). Consequently, the desire for increased productivity in the short term should not be considered greater than the longer term sustainability of the cattle management system (Michel, 1985; Morgan et al., 2012).

Forty-three percent of farmers (n=33) said they administered anthelmintics to cattle and moved them; however it is not known whether this was to new pasture or due to animals being housed. This finding was also highlighted by Barton et al (2006) who reported 42% of their respondents also adopted a 'dose and move' strategy. Dose and move' is a strategy first advocated in 1912 (Thelier, as cited in (Van Wyk, 2001)), the general premise being worms could be eradicated if animals were treated and then moved to pasture that had not been grazed for a year. In this system, any larvae which had previously hatched on this pasture would be unlikely to have survived over the winter period, resulting in a clean pasture for cattle to graze on.

Having been administered with an anthelmintic prior to moving, susceptible nematodes would be removed and the animals would carry a low risk of infection onto these clean pastures (Van Wyk, 2001). This practice has been implicated as a risk factor for anthelmintic resistance because any nematodes surviving anthelmintic treatment will be at a greater prevalence on these previously clean pastures, allowing populations of resistance genes to proliferate at a greater rate than if they were diluted by susceptible populations (Prichard et al., 1980) but this is clearly still being conducted despite best practice advice to the contrary.

In addition to direct parasite control as a result of anthelmintic application, the importance of grazing management should be considered. Only six farmers stated that they used grazing management in addition to anthelmintics for helminth control. Previous publications have highlighted the clear need to integrate parasite control with grazing management (Sangster, 1999), with Barger (1999) proclaiming grazing management to be one of the most crucial aspects of parasite control. Whilst there is a need to prevent parasitic disease, grazing management should also allow for low levels of parasitic infection, in order for immunity to develop in the calves, in addition to providing refugia to slow the selection of anthelmintic resistance (Barger, 1999). To generate a greater awareness of the influence grazing management can exert in a helminth control program, there is a need for greater weight to be given in publications such as SCOPS and COWS and more research on epidemiology of cattle parasites under current farming strategies, given that most epidemiological studies were conducted prior to the release of ML anthelmintics, for example (Armour et al., 1969b; Michel, 1969a). It has been proposed that the primary

responsibility for preserving efficacy of anthelmintics lies with the individual farmer and they should seek advice on use from veterinary surgeons (Coles, 1997). The results here would indicate that whilst a variety of information sources are sought, when asked to rank the most important sources, veterinary surgeons were the most frequent choice. As such, there is a clear role for veterinary surgeons in promoting the sustainable use of anthelmintics; a role which is currently not being fulfilled. This is evident from the findings in this survey, in particular, the lack of effective quarantine practices, the heavy reliance on ML anthelmintics, the lack of anthelmintic class rotation, the treatment of adult cattle and the lack of FEC monitoring to determine treatment requirements. This finding concurs with Morgan et al., (2012), who stated that veterinary surgeons need to be more proactive in providing decision support. The subject of making anthelmintics available only through veterinary surgeons in the UK is currently being discussed (Veterinary Record, p 145, 9 February 2013), with a view to removing the prescribing rights of Suitably Qualified Persons (SQPs) to maintain anthelmintic efficacy. The use of SQPs as an advice source was omitted from this survey; however, in hindsight and in view of recent developments, it would be of use to know how farmers viewed the role of SQPs. In Europe, several countries, such as Denmark and Switzerland, have adopted the prescription only by veterinarians approach, however there is evidence that it has not stopped the development of resistance in these countries and the restriction of product prescription may lead to importation from other countries or a "grey market" of distribution from other sources, such as Internet retailers (Nielsen et al., 2006).

In studies conducted in New Zealand, (Kettle et al., 1981; Kettle et al., 1982) sources of advice on parasite control were grouped as being either of 'commercial' or 'non-commercial' origin. Advice from veterinary surgeons and pharmaceutical representatives were considered amongst the 'commercial' sector, whereas advice from other farmers and the respondents own experience was classed as 'non-commercial'. Articles in magazines were considered to be 50% of each class. If the same approach is taken with this study, the advice sources selected here, would currently be predominantly skewed towards 'commercial' aspects, however if re-categorisation were to occur, the roles of veterinary surgeons may change, and the influence of pharmaceutical representatives may alter.

Further work would be required to ascertain the provenance of meetings being provided for farmers (for example, are they led by veterinary practices or by pharmaceutical industry) as this may affect the bias of advice presented. It is clear that although farmers may consult a variety of information sources, ultimately they make their own decisions. Decisions made with regards to management practices have been described as a compromise between activities they must do to ensure farm survival, those that they would like to do and those that are not achieved (Magne et al., 2012). Farmers willing to undertake preventative measures to control parasite populations have also had their success attributed to this forward thinking approach (Cabaret, 2003), and in beef producers, animal health has been ranked highly as an essential factor in ensuring farm survival (Magne et al., 2012). In addition, it has been reported that farmers trust scientific reports regarding animal disease, as

reported in farming press, but are not always seen as relevant or providing practical advice (Garforth et al., 2013).

Work by Salmona (1994, as cited by (Cabaret et al., 2009)) described decisions in agriculture being based on three types of knowledge: algorithmic (learnt through reading or teaching, for example, at college); memetic (learnt through demonstration, for example, from veterinary surgeons, advisors or other farmers) and phoric (what is felt by the individual, learnt through past experiences). It has been hypothesised that the difference in weight of these three areas will differ considerably between veterinary surgeons and farmers, and needs to be considered when disseminating advice (Saddiqi et al., 2012). From the results shown in this study, it is clear that the effect of meetings is an influence on farmer behaviour with respect to worm control, particularly on beef farms. On a more applied level, Morgan et al (2012) highlight the difficulties faced when proposing changes to parasite control programs; as maximal and sustainable use of anthelmintics are mutually exclusive targets. As a result, long term cost-benefit analysis of changing anthelmintic usage may be influential. An updated version of the study conducted by Michel (1981) to highlight the potential for farmers to maximise their investment in nematode control, by targeting treatments at the most pertinent times of the year, together with analysis on potential productivity losses attributed to the use of ineffective anthelmintics (once resistance has developed) may act as an incentive to commercial farmers, as well as promoting the sustainable use of anthelmintics and the importance of checking efficacy.

An area where this may be more likely to change than most may be in the organic cattle sector. Work by Cabaret (Cabaret, 2003) suggests organic cattle farmers may be more open to alternative preventative measures due to greater motivation to adopt new practices due to inability to use conventional anthelmintics. Greater dissemination of advice was also shown to be an influential factor in Brazil, where the study conducted in 1996 showed that although 95% of respondents were planning to continue with their current control program, nearly all (97%) said they would be willing to alter plans to try new measures of parasite control if they were made aware of them (Charles and Furlong, 1996). This was given further credence by findings of a study (Garforth et al., 2013) where farmer attitudes to implementing biosecurity measures were surveyed and found farmers willing to be convinced about measures they do not use (such as isolating new stock) but needed further supporting evidence from a source seen as credible, such as a trusted veterinary surgeon, and an assessment of relevance as perceived by the individual farmer.

The results here have shown that best practice advice does not appear to be followed universally by Scottish cattle farmers and that beef and dairy farmers implement different approaches to helminth control.. While farmer decisions are clearly determined by a number of factors, the role of veterinary surgeons and SQPs needs to be addressed in order for changes to be made. There is a need for further understanding of the dynamics behind farmer and veterinary interactions so that a cohesive strategy can be defined to benefit the farmer, in terms of maximum benefit from anthelmintic treatments applied to their cattle, whilst maintaining efficacy of anthelmintics used.

Chapter 3: The use of faecal egg count reduction tests to assess ivermectin sensitivity in UK cattle nematodes *

*A proportion of the results from this Chapter have been published in a peer-reviewed journal article, see Appendix 2.

3.1 Introduction

The extent of anthelmintic resistant nematodes in ruminant and equid populations globally requires the standardisation of robust tests to correctly identify resistance to assist farmers and veterinary surgeons in the selection of suitable anthelmintics classes for treatment and control (Kaplan, 2004). Anthelmintic resistance (AR) has previously been described as being a heritable reduction in the relative sensitivity of a nematode population to the action of an anthelmintic (Conder and Campbell, 1995). The faecal egg count reduction test (FECRT) is one of two *in vivo* tests that can be used for the detection of AR in parasitic nematodes and is generally considered to be a relatively straightforward and effective test for the assessment of drug efficacy (Coles et al., 1992). In the FECRT, infected animals are treated at the appropriate dose rate with an anthelmintic and faecal egg count (FEC) analysis is conducted using faecal samples taken on the day of treatment and 14 - 17 days after treatment (Coles et al., 1992). The FECRT is suitable for assessing efficacy of all classes of anthelmintic and in many species of grazing animals (Coles et al., 1992). It also holds an advantage over controlled efficacy tests, in that it does not require necropsy of treated animals to determine the effect of treatment on nematode burden

(Wood et al., 1995). However, a disadvantage of the FECRT is that FEC values are only representative of a patent infection (Michel, 1968). There is also some debate as to whether FECs provide an accurate representation of adult worm burden. A positive correlation between FEC and adult worm burden in cattle has been reported (Bryan and Kerr, 1989); however other studies in cattle have reported a lack of correlation (Michel, 1969e; Brunsdon, 1971; Smeal et al., 1977). FEC values do not provide a quantitative measure of the numbers of developing or inhibited larvae in the abomasal or small intestinal mucosa (Michel, 1963) such that a high FEC is not necessarily indicative of high worm burden and vice versa (Michel, 1969f). Consequently, a FECRT can only estimate anthelmintic efficacy as it relates to egg output of mature female nematodes (Presidente, 1985), which can be indicative of levels of pasture contamination (Kloosterman, 1971). However, in lieu of any other non-invasive detection methods, FECs remain the easiest and cheapest technology for diagnostic purposes (Vercruysse and Claerebout, 2001).

The World Association for the Advancement of Veterinary Parasitology (WAAVP) has developed a series of guidelines for a standardised FECRT (Coles et al., 1992; Wood et al., 1995; Coles et al., 2006); however, these guidelines are designed primarily for use in sheep and goats, with optimised tests for AR detection in cattle nematodes currently unavailable (Sutherland and Leathwick, 2011). Within the published guidelines, there are many points that are the subject of debate (van Wyk and Groeneveld, 1997) and it is recognised that there needs to be a degree of flexibility when testing anthelmintic products in different host species (Wood et al., 1995). Discussions surround issues such as the number of animals to be tested, the

use of control groups and the use of composite, or pooled, faecal samples (Coles et al., 1992; Ward et al., 1997; Presland et al., 2005; Coles et al., 2006; McKenna, 2007). Amongst the most debated, however, is the determination of which FEC method is most suitable for a particular host species and the associated downstream statistical analysis of the data from which sensitivity is inferred (Presidente, 1985; Dash et al., 1988; Presland et al., 2005; Torgerson et al., 2005; Rinaldi et al., 2011; Dobson et al., 2012; Levecke et al., 2012; Torgerson et al., 2012).

From the questionnaire data presented in Chapter 2, it would appear that many farmers on UK beef farms administer anthelmintics to cattle on average twice a year, usually to coincide with turn-out in spring and housing in late autumn and winter. It was noted that farmers prefer to use macrocyclic lactone (ML) anthelmintics, particularly pour-on applications. This can be attributed to a combination of the greater efficacy of ML anthelmintics (González Canga et al., 2009) and the convenience of topical applications (Brooker and Goose, 1975). These findings concur with those of a previous small questionnaire study conducted in the south-west of England (Barton et al., 2006); however, the effectiveness of the anthelmintics used on these farms was unknown. Information regarding the prevalence of different nematode species on UK cattle farms is largely based on studies conducted prior to the widespread use of broad-spectrum anthelmintics and studies relating to this fall into two major categories. First, those that investigated the epidemiology of parasitic nematodes, predominantly published before 1980, and which focused on standardisation of helminthological nomenclature (Oldham, 1938) and confirmation of species not previously identified in the UK (Baylis, 1938; Morgan and Soulsby,

1956). Studies of nematode species present on pasture and the pathogenicity of these species in cattle were often published in the form of clinical case reports (Michel, 1963, 1969a, f), abattoir studies (Bairden and Armour, 1981) and examination of pasture contamination (Bairden et al., 1985).

IVM resistance in cattle nematodes in the UK was first reported in *Cooperia* spp. (Coles et al., 1998; Stafford and Coles, 1999) in a study where dairy calves on a farm in Somerset were reported to be in poor condition and subsequently treated with an injectable IVM product. On two successive occasions, faeces were found to be positive for nematode eggs seven days after treatment, with a mean FECR of 65% recorded. Following necropsy of two of the calves, worm burdens predominantly comprised *Cooperia* spp. (88%) and *Nematodirus helvetianus* (12%). *Cooperia* spp. larvae were also cultured from faeces obtained from calves on the farm and were used to infect eight, helminth-naïve calves under controlled experimental conditions and an efficacy test was performed (Stafford and Coles, 1999). Four calves were treated with injectable IVM at the recommended dose rate of 0.2 µg kg^{-1} body weight. Calves were necropsied seven days after treatment and only a 16% reduction in *Cooperia* spp. worm burdens was observed compared to untreated control calves. As a result, the first report of IVM (and hence ML) resistance in *Cooperia* spp. in the UK was confirmed. A second report followed, in which FECs were reported to have increased when FEC analysis was performed 21 days after administration of injectable IVM (Coles et al., 2001). A mean FEC of 155 eggs per gram (EPG) of faeces was reported and *Cooperia* spp. were present in larval cultures derived from faeces from the treated calves (Coles et al., 2001). Following this, reports of ML

inefficacy have been predominantly restricted to small clinical studies, where anthelmintic failure was suspected (Coles et al., 2001; Sargison et al., 2009; Sargison et al., 2010). In 2009, there was a report of a lack of efficacy after administration of DOR pour-on product to cattle on two farms (Sargison et al., 2009). On both farms, Highland calves were observed to be passing loose faeces following DOR pour-on treatment. Subsequent administration of an injectable formulation of DOR was 100% efficacious in reducing trichostrongyle FECs on both farms and the previous presumed reduced efficacy attributed to the method of application (Sargison et al., 2009). A report followed in 2010 where a DOR pour-on application was observed to fail in preventing nematode infection of first season grazing (FSG) calves grazed for five weeks prior to housing (Sargison et al., 2010). The calves were reported to be in poor condition, FECs increased whilst housed and larval cultures indicated the presence of *Cooperia* spp. In another study (Coles et al., 2008), faecal samples were analysed from 16 farms on the day of anthelmintic administration, and again, 14 days later. Here, a sensitive FEC method (FLOTAC; (Cringoli, 2006)) was employed following treatment and FECR values after pour-on IVM administration ranged from 0 to 99% (10 farms: 0, 0, 37, 40, 73, 38, 89, 91, 92 and 99% efficacy); 43 to 100% after injectable IVM administration (two farms: 43 and 100% efficacy) and 91 to 98% after pour-on MOX administration (three farms: 91, 97 and 98% efficacy). However, as this study was conducted using different FEC methodologies, comparisons of FEC before and after anthelmintic administration may not be statistically valid. Together, these studies would appear to suggest the presence of anthelmintic-resistant *Cooperia* species in the UK.

It is clear that there is relatively little recent information regarding the efficacy of ML anthelmintics in the UK cattle population. The aim of this chapter is, first, to examine the sensitivity of IVM in nematode populations in FSG calves on a cohort of 20 UK farms and the changes in nematode species prevalence as a result of anthelmintic administration. The second aim is to examine the impact of FEC methodology on the outcome of the efficacy testing. Parameters examined include a comparison of two FEC methodologies of varying sensitivity (1 EPG *versus* 50 EPG), comparison of utilising arithmetic or geometric means, and a comparison of different analytical methods used to calculate efficacy values. By examining the impact of these parameters on the outcome of FECR testing, the objective is to develop a robust method for investigating nematode anthelmintic sensitivity *in vivo* in cattle.

3.2 Materials and methods

3.2.1 Participating farms and sample collection

Farmers wishing to participate in the IVM FECRT were identified following the questionnaire mailing to Moredun Foundation members, as described in Chapter 2 (Section 2.2.1). All interested parties were issued with an information sheet, describing the FECRT procedure in detail. Farmers were then contacted via telephone or email to discuss the sample collection procedure. At this time, farmers were asked to provide details of the next time they would expect to administer anthelmintics to their first season grazing (FSG) calves and if they had access to a weigh crate and animal handling facilities. Approximately two weeks prior to the anticipated anthelmintic administration date, a sampling kit was posted to farmers.

Items within the kit included:

- injectable IVM (Ivomec Super®, 1% (w/v) IVM, 10% (w/v) clorsulon, Merial Animal Health, 0.2 µg kg^{-1} body weight; BW),
- 'Ziploc' polythene sample bags (300 mm x 200 mm; Gripwell)
- disposable syringes and sterile needles (17 ½" gauge) (Dunlops Veterinary Supplies);
- cattle weigh tape (Coburn Company, Inc) if required.
- mailing packaging with Freepost labels
- letter reiterating IVM administration and sample collection protocol.

Participants were asked to randomly identify 10 to 15 FSG calves that had been grazing on pasture for more than six weeks prior to the anticipated anthelmintic administration date. On day of treatment (Day 0), they were asked to collect a "fresh as possible" sample from each calf and place the sample in a Ziploc bag, labelled

with the animal number. Then, they were asked to administer the IVM subcutaneously at the manufacturer's recommended dose rate of 0.2 μg IVM kg^{-1} BW. As much air as possible was to be excluded from the sample bags, before being sealed and posted to Moredun Research Institute in the supplied packaging. As far as possible, samples were processed on day of arrival, with all faecal samples processed within 48 h of arrival.

3.2.2 Parasitological techniques

3.2.2.1 Faecal egg count method

For all samples obtained from all farms, FEC analysis was conducted in duplicate using the following centrifugal flotation method adapted from Jackson (1974). Faecal samples were thoroughly mixed and 10 g subsample removed into a plastic bag. To this, 100 ml tap water were added and the contents fully homogenised. A 10 ml aliquot was removed and passed over a 1 mm sieve into a 250 ml beaker and washed through with 5 ml tap water. The remaining 90 ml suspension was mixed again and the process repeated for a second 10 ml aliquot. The beaker contents were poured into 15 ml polyallomer tubes (Beckman Coulter) and centrifuged for 2 min at 203 *x g*. The supernatant was removed using a vacuum line, the tubes refilled with saturated sodium chloride solution (NaCl, specific gravity 1.2) and gently inverted several times to resuspend the faecal pellet. The tubes were centrifuged for a second time for 2 min at 203 *x g*. Artery forceps were then used to clamp the tubes approximately 0.5 cm below the meniscus of the supernatant and this portion of the supernatant (containing the nematode eggs) was poured into a 2.5 ml spectrophotometer cuvette (Fisher Scientific). Additional NaCl solution was added to rinse the upper portion of the tube and added to the cuvette. A lid was placed on

the cuvette and the latter then placed on a stereomicroscope stage. To aid enumeration of eggs, a Miller Square graticule was used (Graticules Ltd, UK). If the number of eggs present were at low density (for example, less than 50 eggs per gram (EPG)), all of the eggs in the cuvette were counted. Under these circumstances, one egg counted is equivalent to one EPG faeces. If the eggs were deemed to be of moderate density (for example, between 50 and 100 EPG), then the eggs were counted by making two traverses of the cuvette, counting the eggs which appeared in the larger square in the graticule, as demonstrated in Figure 6(A). After two traverses, the number of eggs counted were added together and multiplied by three to give the equivalent EPG. Similarly, when the number of eggs appeared to be in excess of 100 EPG, two traverses were made, counting the eggs which appeared in the smaller of the graticule squares, demonstrated in Figure 6(B). As this is equivalent to counting one ninth of the cuvette, the number of eggs counted were added together and multiplied by nine to give the EPG. As two cuvettes were counted for each sample, both EPG were recorded and an arithmetic mean was taken of the two counts. All enumeration was conducted at x40 magnification.

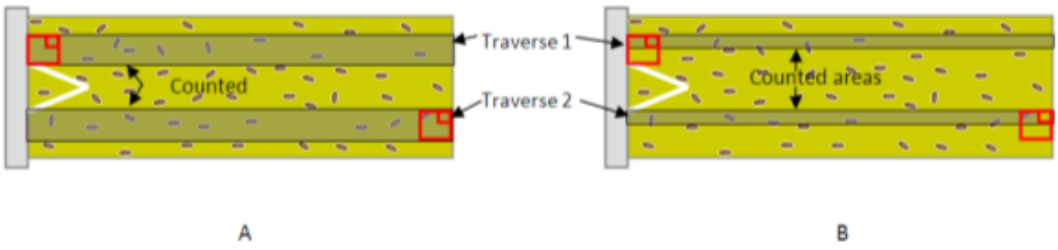

Figure 5: Use of Miller square graticule when counting nematode eggs using the adapted FEC method (Jackson, 1974). The cuvette in the left hand pane (A) demonstrates two traverses with the large square. All eggs that fall within this square are recorded over two traverses, are added together and multiplied by three to give the number of eggs per gram. The cuvette in the right

pane demonstrates the protocol when counting at higher egg density with the smaller square; the number of eggs counted being multiplied by nine following two traverses. (Image reproduced with permission by Dr David Bartley, Moredun Research Institute

3.2.2.2 McMaster faecal egg count method

To allow results from this study to be directly comparable to those from other FECRT studies, faecal samples from 16 farms (005 – 024) were also subjected to McMaster FEC analysis. The protocol followed was adapted from that of Gordon and Whitlock (1939). For each calf, a 3 g subsample was removed from the homogenised faecal sample and placed into a plastic bag. To this, 42 ml saturated NaCl solution (specific gravity 1.2) were added, the suspension thoroughly mixed and poured over a 1 mm aperture sieve into a 250 ml plastic beaker. The suspension was mixed and a 3 ml Pasteur pipette (Sterilin) used to withdraw 1.5 ml faecal solution. This was used to rapidly fill two chambers of a McMaster counting slide (Chalex Corporation, UK). The remaining solution was mixed again before a further 1.5 ml aliquot removed and used to fill two chambers of a second McMaster counting slide. The slides were enumerated under x 40 magnification, and the number of eggs observed under each counting grid was noted. For each slide, the number of eggs observed under the two counting grids were added together and multiplied by 50 to give the number of EPG of faeces (Gordon and Whitlock, 1939). The two EPG values were averaged to produce a mean McMaster EPG.

3.2.2.3 *Dictyocaulus viviparus* enumeration using the Baermann method

To ascertain the presence of *Dictyocaulus viviparus* larvae, an existing protocol was adapted (M.A.F.F., 1986). A modified Baermann apparatus was constructed using a small catheter tap connected to the tip of a 50 ml syringe. The syringe was held upright in a stand, with the tap closed and pointing down. The plunger was removed and the barrel filled with tap water. For each animal, 10 g faeces were removed from the homogenised sample, and enclosed in two layers of muslin, secured with a wooden cocktail stick. This was placed into the syringe barrel, with tap water added as necessary to ensure the faeces were fully submerged, and incubated overnight at 20°C. The next day, the tap was opened and the *D. viviparus* larvae collected into a 50 ml Falcon tube (Sterilin). The Falcon tubes were then centrifuged at 203 *x g* for 2 min, the supernatant removed to leave a volume of approximately 2 ml. This was transferred to a Petri dish and examined under x 40 magnification.

3.2.2.4 *Fasciola hepatica* egg sedimentation method

For the examination of faecal samples for the presence of *Fasciola hepatica* eggs, the following protocol was used (McCaughy and Hatch, 1964). For each homogenised sample, 3 g of faeces were removed into a plastic bag, to which 42 ml tap water were added and thoroughly mixed to an even suspension. The faecal suspension was poured over a 150 µm aperture sieve (Fisher Scientific) into a 250 ml inverted conical measuring cylinder (McKay and Lynn Ltd). A further 125 ml tap water was added to the sieve to wash into the cylinder. The cylinder was left to stand at a 45 degree angle for 3 min at 20 °C, to allow the eggs to sediment. After this time, the

supernatant was removed via vacuum line, the sediment stained with one drop of methylene blue (2% w/v, Sigma Aldrich) and transferred to a Petri dish (Sterilin). The Petri dish contents were examined on a dissecting microscope at x 40 magnification. For each animal, the number of *F. hepatica* eggs counted was divided by three to give the number of *F. hepatica* EPG of faeces.

3.2.2.5 Fluorescent peanut agglutinin (PNA) lectin staining method

A fluorescent peanut agglutinin lectin staining method was used to determine the presence of *Haemonchus* spp. eggs in submitted samples. Faecal suspensions remaining from the nematode CF FEC method (approximately 80 ml per animal) were pooled from all animals on one farm and washed over a series of sieves, decreasing in pore diameter (1 mm, 500 µm, 212 µm, 125 µm and 38 µm; Fisher Scientific). Collected eggs were retained on the 38 µm sieve, poured into polyallomer tubes and centrifuged for 2 min at 203 *x g*. The supernatant was removed, tubes refilled with saturated NaCl solution and centrifuged for a further 2 min at 203 *x g*. The tubes were clamped with artery forceps and the recovered eggs washed over a clean 38 µm sieve. The eggs were rinsed with tap water to remove residual salt and transferred to a clean centrifuge tube. Five aliquots of 10 µl nematode egg suspension were counted under x 100 magnification and the concentration of eggs adjusted to one egg per ml. The 'clean' nematode egg solution was then used in an adapted lectin staining method (Palmer and McCombe, 1996). A working solution of PNA was made by adding 10 µl PNA stock solution (PNA 5 mg

ml^{-1}; 10 mM HEPES, 0.15 M sodium chloride, 0.08% sodium azide, 0.1 mM calcium; Vector Laboratories) to 990 µl PBS (phosphate buffered saline) and vortexed briefly. To 900 µl egg suspension (approximately 900 eggs), 100 µl working solution PNA were added (final concentration 0.5 µg ml^{-1} PNA) and incubated for 1 h at 20 °C in darkened Eppendorfs (Axygen). A separate monospecific suspension of *Haemonchus contortus* eggs (kindly provided by Mrs Alison Morrison, Moredun Research Institute) were incubated to provide a positive control. After 1 h, eggs were transferred to a glass slide for counting and examined under x 100 magnification using an inverted fluorescence stereomicroscope fitted with a UV blue range filter (495 nm).

3.2.2.6 Culture of faeces and larval extraction for species differentiation

With the exception of Farm 001, equal quantities of faeces from all animals (Farms 002 – 024) were pooled for culture to provide third stage larvae (L_3) for morphological identification. Due to the small sample sizes submitted, all excess faeces from animals from Farm 001 were pooled and cultured. The faeces were mixed with equal volumes of vermiculite, formed into balls approximately 5 cm in diameter, placed into plastic trays, covered with perforated polythene bags to allow air circulation and incubated at 22 °C for 14 - 17 days. This was conducted for samples obtained on both Day 0 and Day 14 following IVM treatment. A coproculture was not conducted on Day 14 samples from Farm 002, due to zero FEC, as in keeping with previously published research (Waghorn et al., 2006; Demeler et al., 2009; El-Abdellati et al., 2010a), however for all other farms, both sets of

samples were cultured, regardless of FEC. This was conducted to investigate if samples with a zero FEC would yield any larvae once cultured. If larvae were found, this would indicate the FEC result was not truly zero, with the potential for trichostrongyle eggs to have survived IVM administration. After incubation, trays were flooded with tepid tap water and incubated for 4 h at 22 °C. The supernatant was then poured over a 1 mm sieve into a 10 l bucket and the faeces discarded. The supernatant was incubated overnight at room temperature, the volume reduced to less than 1 l and transferred to a plastic jug. Next, L_3 were collected by a modification of the Baermann technique (Baermann, 1917). A Baermann device was constructed by securing two sheets of Whatman© filter (GE Healthcare Companies) paper between two plastic collars. The paper was moistened with tap water and the contents of the jug slowly poured through the device. A 400 ml glass jar was filled with tepid water and the device placed into the neck of the jar, and left overnight at room temperature. The next day, the Baermann device was removed and the volume reduced via a vacuum line to give approximately 20 ml larval culture. The larval suspension was transferred to vented culture flasks (Corning) and the L_3 identified to genus level using morphological keys (M.A.F.F., 1986) and with reference to previously published studies (Hansen and Shivnani, 1956). For each sample, 100 randomly selected L_3 were examined at x 100 magnification.

3.2.3 Statistical analysis

Data was recorded and arithmetic and geometric calculations performed in Microsoft Excel 2007. Larval genus results were subjected to a test of equal proportions, to

ensure that the proportions of larvae surviving passage were similar to those originally found on farm ("prop.test"; (Newcombe, 1998)), and together with the parametric bootstrapping analyses, conducted in R statistical environment (version 3.0.1), using R Studio (version 0.97.551). Graphs were drawn using Minitab 15 (Minitab Inc, 2006) and Microsoft Excel 2007 and with the use of 'ggplot2' package in R (Team, 2013).

3.2.3.1 Arithmetic and geometric mean calculations

The current WAAVP method for calculating FECR value (Coles et al., 1992) does not account for multiple counts made from one faecal sample. Here, FECs were conducted in duplicate for each sample, resulting in the generation of two EPG values per animal. To examine this effect on FECR calculation, the data was initially analysed in two ways. First, as every animal had two EPG counts, each EPG value generated was treated as a separate count; for example, samples from 10 animals resulted in a total of 20 EPG values. Using this method, all data collected went forward to the FECR calculation, referred to as "raw". For the second method, an arithmetic mean of the two EPG values was made, and this combined value went forward to the overall FECR calculation, referred to as a "combined" value. Unless explicitly stated, the "combined" mean was used to generate arithmetic and geometric means in the remainder of the statistical analyses. Overall, arithmetic means were calculated from datasets from each farm by adding the FEC values together and dividing by the number of samples present. Geometric means were also calculated using the GEOMEAN function in Microsoft Excel, which calculated the anti-log of log-transformed counts (Dash et al., 1988). In order to calculate geometric means, a nominal value of one was added to each EPG prior to analysis,

and subsequently removed following analysis, as accepted practice (Fulford, 1994). There is no universal standard for the value that is required to be added prior to analysis (Fulford, 1994). The addition of a nominal value serves to address any zero EPG values in the dataset and is subsequently removed following analysis, to avoid influencing the final FECR result. In addition, for the McMaster method-generated data, a value of 25 was added to each EPG value (half the minimum detection level of a McMaster count), as suggested by Dash (1988). The formula used was as follows:

$$\text{Geometric Mean} = ((X_1)(X_2)(X_3).....(X_n))^{1/n}$$

Where 'n' is the number of calves tested and 'X' is the "raw" or "combined" FEC for a particular sample.

In order to examine the degree of aggregation (i.e. clumping) of FEC prior to anthelmintic administration, k values were generated, by examining the estimated variance to mean ratio of the samples. Here, k values equal to one indicate the variance is equal to mean, indicating a Poisson (random) distribution (Shaw and Dobson, 1995). Values of less than 1 indicate a highly aggregated data set. The calculation used is a corrected moment estimate (Smith and Guerrero, 1993), as follows:

$$k = m^2 – (v/n)/ v – m$$

Where 'm' is mean FEC, 'v' equals the variance between the FECs of selected samples and 'n' is the number of animals tested.

3.2.3.2 Standard faecal egg count reduction calculation

Following data processing, FECR was calculated using the following formula (Kochapakdee et al., 1995):

$R = 100 \times ([T_1 - T_2]/T_1)$

where 'R' is the FECR value, 'T_1' is the mean FEC on day of treatment, and 'T_2' is mean post-treatment FEC. This calculation is similar to that published in the WAAVP guidelines (Coles et al., 1992) but does not include FECs derived from untreated control groups. The 95% confidence intervals were calculated as described by (Anderson et al., 1991) and (Coles et al., 1992). Any negative values (observed, for example, if FEC values increased following treatment) were corrected to zero (McKenna, 1990) and all values rounded to the nearest integer. This process was used for FECs generated by centrifugal flotation and McMaster methods and for data generated by calculating the geometric and arithmetic means of the FECs. This calculation, when used with the "combined" FEC data, was used as the reference calculation to compare all other methods of analysis, marked as [WAAVP].

3.2.3.3 Parametric bootstrapping

A comma separated values (.csv) file was generated from the FECRT dataset comprising four columns, namely Farm ID, the arithmetic mean Day 0 and Day 14 EPG values for each farm and the number of animals sampled from each farm. This file was imported into Rstudio and an empty four-dimensional array constructed. For

all 20 farms. there were 20 rows (to contain the Day 14 FEC values for each farm), 20 columns (to contain the maximum number of Day 0 FEC values, as the maximum number of calves sampled was 20), and a dimension to fit 10, 000 bootstrapping iterations. Next, values from the Poisson distribution (Hunter and Quenouille, 1952) were randomly generated from Day 0 values, 10,000 times for each farm, with sampling length limited to the number of individual samples from a particular farm. The Poisson distribution was chosen because although FEC within a group of cattle are thought to be negative binomial distribution, FEC within a sample were found to fit Poisson distribution. Next, 10, 000 bootsrapping iterations were run for each of the Day 0 values. Then,, a FECR calculation (as described in Section 3.2.3.2; (Kochapakdee et al., 1995) was performed using the bootstrapped Day 14 values by Day 0 values (10, 000 calculations performed in total, for each farm) to get a percentage efficacy value and this was added to the array. The fourth dimension of the array was flattened to allow graphs to be drawn, and mean, median and 95% confidence interval values were generated for each farm and exported as a .csv file. This was conducted for both centrifugal flotation and McMaster derived data sets.

3.2.3.4 Determination of ivermectin resistance

For all methods of analysis, IVM resistance was indicted where the mean FECR was less than 95%, with a lower 95% confidence interval of less than 90% (Coles et al., 1992). If only one of these two criteria was met, then IVM resistance was suspected. Hereafter, farms indicated as “R” met both criteria for IVM resistance, farms marked as “S” met neither criteria (i.e. IVM sensitivity indicated) and farms marked as “S?” showed a FECR value of 95% or greater, but had a lower 95% confidence interval of less than 90%.

3.2.4. Analysis of risk factors for IVM resistant nematodes

All farms conducting the FECRT had completed the questionnaire described in Chapter 2. In order to elucidate potential differences in parasite control and management strategies between farms considered to have IVM resistant or sensitive nematodes, univariable logistic regression analysis was conducted. Analysis was conducted as described in Chapter 2, Section 2.2. Briefly, a column ("IVM resistant") was added to the existing csv. file and the resistance status of each farm added. The FECR classification result used for this purpose was that of the AM analysis conducted with the CF methodology. The outcome "IVM resistant" was then tested against all areas of best practice advice parameters described in the COWS (Control of Worms Sustainably) manual (EBLEX, 2010).

3.3 Results

3.3.1 General descriptive results

Participating farms were located across the UK, with five farms located in England and 15 farms in Scotland. Postal codes provided by respondents were entered into publically available, open-source map generation software (www.batchgeo.com) to create the map shown in Figure 7.

Figure 6: Map of participating farms, generated from farm postal codes

FECRTs were undertaken in the autumn and winter periods of 2010 (Farms 001 – 004), 2011 (Farms 005 – 019) and 2012 (Farms 020 – 024). In total, 20 farms completed a FECRT, with Farm 004 supplying samples from two cohorts of FSG calves: 004a were calves grazed with their dams since birth, with calves from 004b grazed on different pastures and turned onto pasture one month later. All calves tested had been born in the spring of that year and had grazed over the preceding

summer months. One farmer (Farm 009) chose to use pour-on IVM (Ivomec Classic® Pour-On, 0.5% (w/v) IVM, Merial Animal Health, 0.5 mg/kg BW) on all animals in the test group and at Farm 015, pour-on IVM (Ivomec Classic® Pour-On, 0.5% (w/v) IVM, Merial Animal Health, 500 µg/kg BW) was administered to 7 animals and injectable IVM (Ivomec Super®, 1% (w/v) IVM, 10% (w/v) clorsulon, Merial Animal Health, 0.2 µg IVM kg^{-1} BW) to a further seven animals. For farms 001, 008, 010, 015, 020 and 021, the farmers were issued with a weight tape to estimate the weight of the calves, and on the remaining farms, calves were weighed on weigh crates. Information regarding weight data (obtained using crate scales) and volume of anthelmintic administered to each calf was volunteered from farmers on Farms 002, 004 and 015. A further five farmers volunteered electronic identification tag output from weight scales.

3.3.2 Basic parasitological analysis on samples derived from all test farms

All faecal samples (n = 260) predominantly contained trichostrongyle eggs. The presence of *Moniezia benedeni* eggs were recorded in nine calves in low numbers. As neither the CF nor the McMaster FEC methods are thoroughly validated as a quantitative detection method for *Moniezia* spp., detailed results are not presented here. The distribution of trichostrongyle egg counts obtained using the CF method are shown in Figure 8 for all pre-treatment samples (n = 260). The majority of FEC values (i.e. 90%) were below 150 EPG, with only 26 animals having FECs in excess of 150 EPG. No trichostrongyle eggs were detected in faecal samples obtained from 16 calves and in samples from 61 animals, fewer than 10 EPG were enumerated.

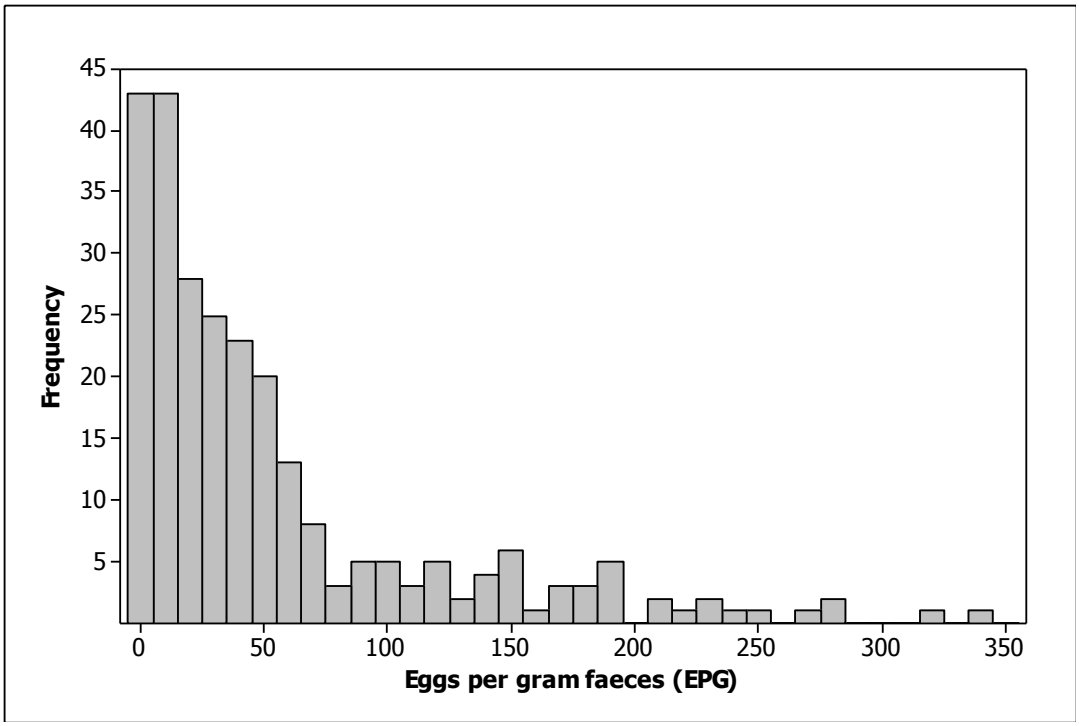

Figure 7: Distribution of trichostrongyle FEC from all calves (n = 260) prior to IVM administration. Data was generated using CF methodology and is presented as "combined" mean FEC for each sample tested.

When FECs were split by year of submission (2010, 2011 or 2012), similar distributions were observed (Figure 9). The mean FEC levels per year were not found to be significantly different between years ($p>0.05$, (Kruskal and Wallis, 1952).

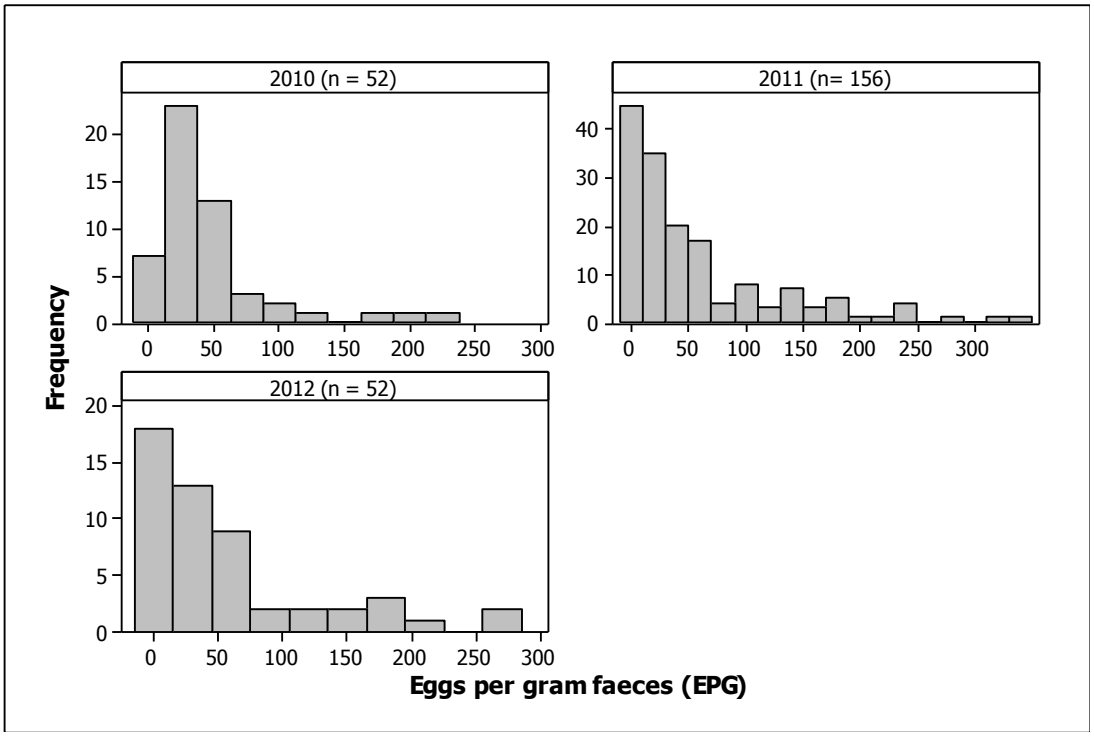

Figure 8: Distribution of trichostrongyle FEC prior to IVM administration by year. Number of samples submitted per year is indicated by n. Data was generated using CF methodology and is presented as "combined" mean FEC for each sample tested.

The k value generated for all 260 samples submitted on day of anthelmintic administration was 0.69, indicating that the FEC dataset was highly aggregated. When analysed for each year, k values of 0.91, 0.65 and 0.67 were generated for years 2010, 2011 and 2012, respectively. The higher k value generated from the 2010 dataset is due to the fewer zero FEC results compared to the two following years.

D. viviparus larvae were not observed in any samples obtained on Day 0 (n = 260), and so the analysis was not conducted on samples obtained after IVM treatment. Similarly, PNA lectin stains were negative for all samples (n = 21), indicating that *Haemonchus* spp. eggs were not observed in these samples. Low numbers of *F.*

hepatica eggs were observed in three samples prior to treatment, from Farms 006, 010 and 019, with a maximum of 3 EPG observed in one sample from Farm 019. No *F. hepatica* eggs were observed in samples obtained after anthelmintic treatment (n= 260).

All submitted samples were analysed for trichostrongyle eggs using the CF FEC method (Section 3.2.2.1). The majority of FECs analysed on Day 0 were below 100 EPG, with the mean FEC across all samples 54 EPG (± 4 standard error of mean; SEM). At farm level, mean FEC ranged from 3 EPG (Farm 008) to 145 EPG (Farm 013). The FEC values differed among farms and, as suggested by the level of aggregation indicated above, samples submitted from the same farm had a wide range of FEC values (Figure 10).

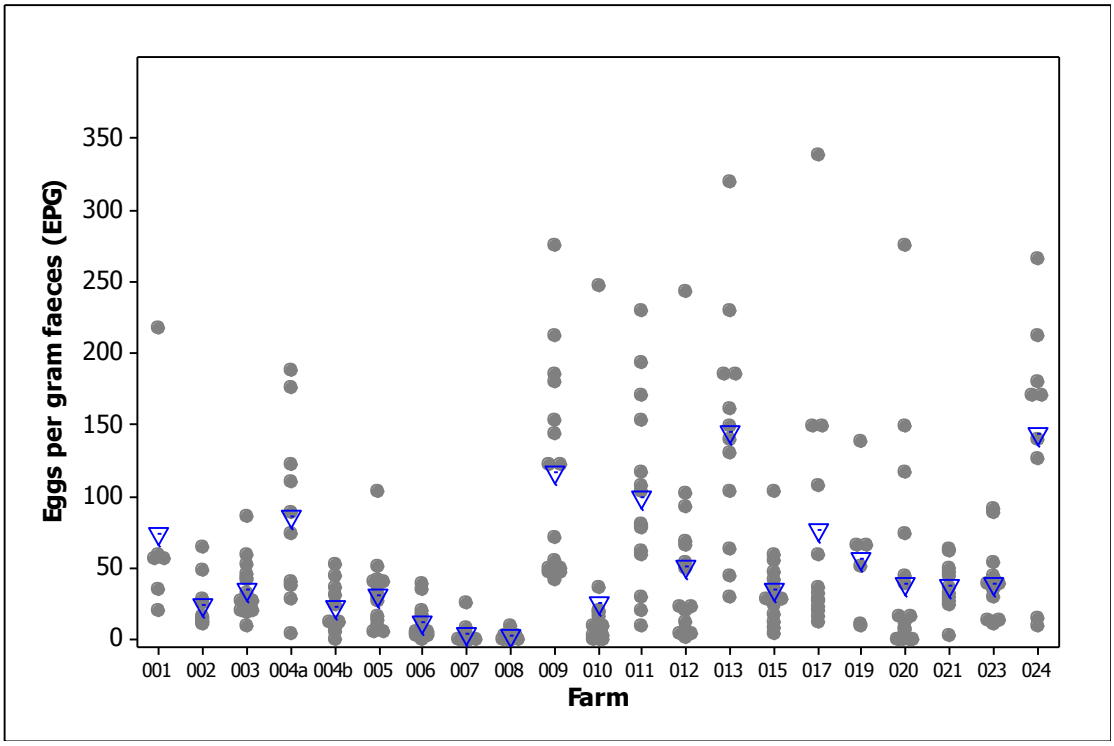

Figure 9: Trichostrongyle FEC values per gram of faeces taken on day of treatment with IVM, for each farm tested. The grey markers indicate FEC of each individual and the blue triangles indicate the mean EPG for each farm. All samples were analysed in duplicate using the CF FEC method.

The FEC data derived from samples submitted 14 days after IVM treatment are displayed in Figure 11. Zero eggs were detected in all samples from Farms 002, 006, 008, 010 and 021 and the mean FEC across all post-treatment samples was 12 EPG (± 2 SEM). At farm level, the highest mean FEC was observed with samples from Farm 013, with a mean FEC of 71 EPG (± 24 SEM). As with the Day 0 samples, FEC values varied between farms and between individuals at farm level. Sixteen farms had at least one zero FEC following IVM treatment. The largest range in FEC on a single farm was observed in samples from Farm 023, where FECs ranged from 2 to 248 EPG. With respect to Farm 015, there was no significant difference observed in FECs at Day 14 after treatment between samples submitted from the seven calves that were treated with pour-on IVM compared to the seven animals that had been administered with injectable IVM.

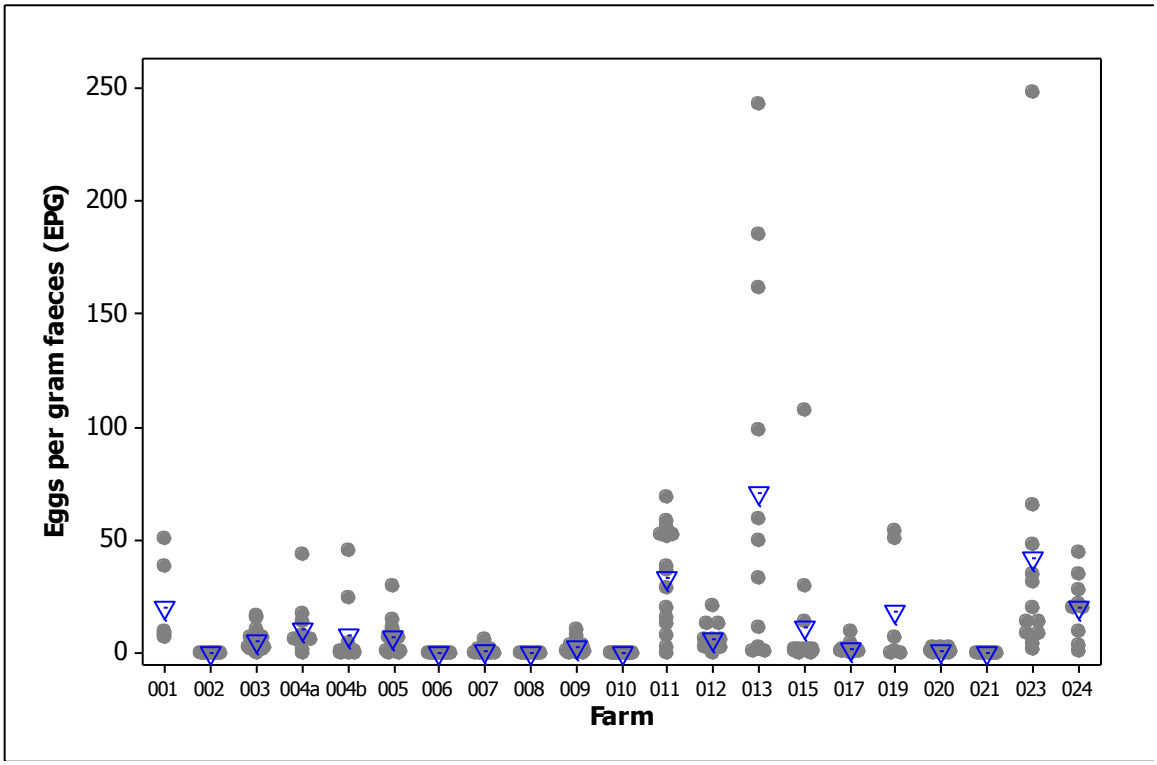

Figure 10: Trichostrongyle FEC values per gram of faeces taken 14 days following IVM administration, for each farm tested. The grey markers indicate FEC of an individual animal and the blue triangles indicate the mean EPG for each farm as measured by the CF method. All samples were analysed in duplicate.

3.3.3. Assessment of efficacy of IVM

Arithmetic mean reductions in FEC were calculated using the formula described in Section 3.2.3.2 and the results displayed in Figure 12. Based on criteria published by WAAVP (Coles et al., 1992), where a declaration of ML resistance is dependent upon a mean FECR of less than 95% and a lower 95% CI of less than 90%, thirteen of the twenty populations tested showed indications of IVM resistance.

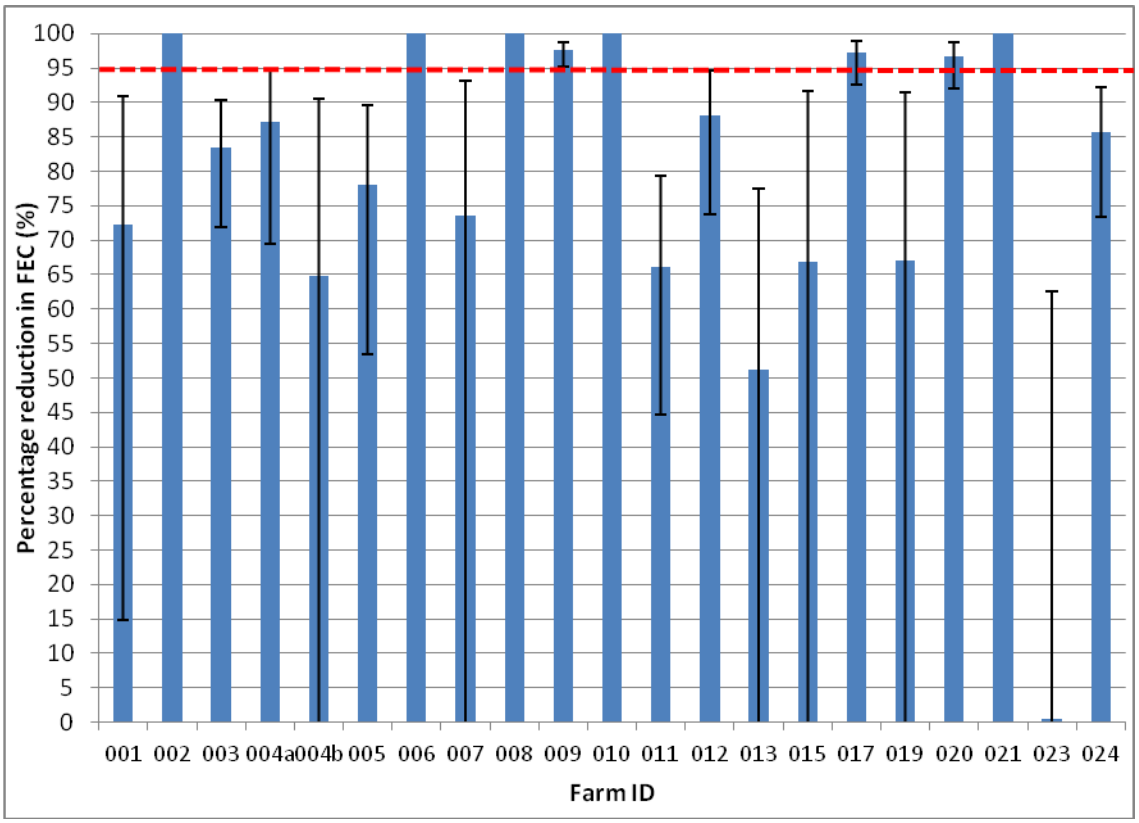

Figure 11: FECRT values per farm. The blue bars represent the mean percentage reduction in FEC for each farm 14 days after IVM treatment. Error bars represent the 95% confidence intervals. The red dashed line indicates the FECR threshold for resistance as determined by WAAVP (Coles et al., 1992).

3.3.4 Identification of third-stage larvae before and after IVM treatment

Culture of pooled faecal samples was undertaken to allow identification of trichostrongyle L_3 to genus level to monitor any change in percentage genus composition following IVM administration (MAFF 1986). One hundred L_3 were identified from all Day 0 samples (n= 21), despite low FEC levels in some samples. Nineteen Day 0 cultures contained a combination of *Ostertagia* spp. and *Cooperia*

spp. L_3 (Table 12). One culture (Farm 008) was comprised entirely of *Cooperia* spp. L_3, and cultures from three farms (Farms 006, 010 and 013) contained *Trichostrongylus* spp. L_3. Following IVM administration, there were changes in the proportions of trichostrongyle L_3. *Cooperia* spp. dominated these cultures (range: 96 - 100%) and lower proportions of *Ostertagia* spp. L_3 (range: 1 – 4%) were present. No *Trichostrongylus* spp. were found to be present following IVM administration. Cultures were also conducted on samples from farms where no eggs were observed following IVM treatment (Farms 006, 008, 010 and 021) and these failed to yield any L_3. From all other farms, a minimum of 100 L_3 were selected from each pooled culture and identified and the results displayed in Table 12, Figures 13 and 14.

Table 12 : Percentage genus composition of trichostrongyle L_3 following faecal culture on samples obtained on the day of treatment (Day 0) and 14 days after IVM administration. Identification was performed using morphological keys (Hansen and Shivnani, 1956; M.A.F.F., 1986) on 100 randomly selected L_3 from all samples from each farm. No Trichostrongylus spp. were observed at Day 14. SNA = samples not analysed. Samples from Farm 002 following IVM administration were not cultured due to zero FEC, however in order to investigate potential for eggs to be present in 'negative' samples, cultures were conducted for all other farms, regardless of FEC.

Farm ID	Day 0 (%)			Day 14 (%)	
	Ostertagia spp.	*Cooperia* spp.	*Trichostrongylus* spp.	*Ostertagia* spp.	*Cooperia* spp.
001	15	85	0	4	96
002	22	78	0	SNA	SNA
003	16	84	0	0	100
004a	38	62	0	0	100
004b	29	71	0	0	100
005	19	81	0	0	100
006	33	63	4	0	0
007	15	85	0	3	97
008	0	100	0	0	0
009	30	70	0	0	100
010	44	3	53	0	0
011	20	80	0	3	97
012	28	72	0	1	99
013	11	81	8	0	100
015	4	96	0	0	100
017	26	74	0	2	98
019	5	95	0	2	98
020	14	86	0	0	100
021	10	90	0	0	0
023	17	83	0	0	100
024	8	92	0	0	100

Results from the proportion test analysis revealed a statistically significant change in *Ostertagia* spp. in cultures from all farms following IVM administration ($P < 0.05$), with the exception of Farm 019. There was a statistically significant change in *Cooperia* spp. prevalence in cultures on all farms following IVM administration, with the exception of Farms 010, 015 and 019 ($P > 0.05$).

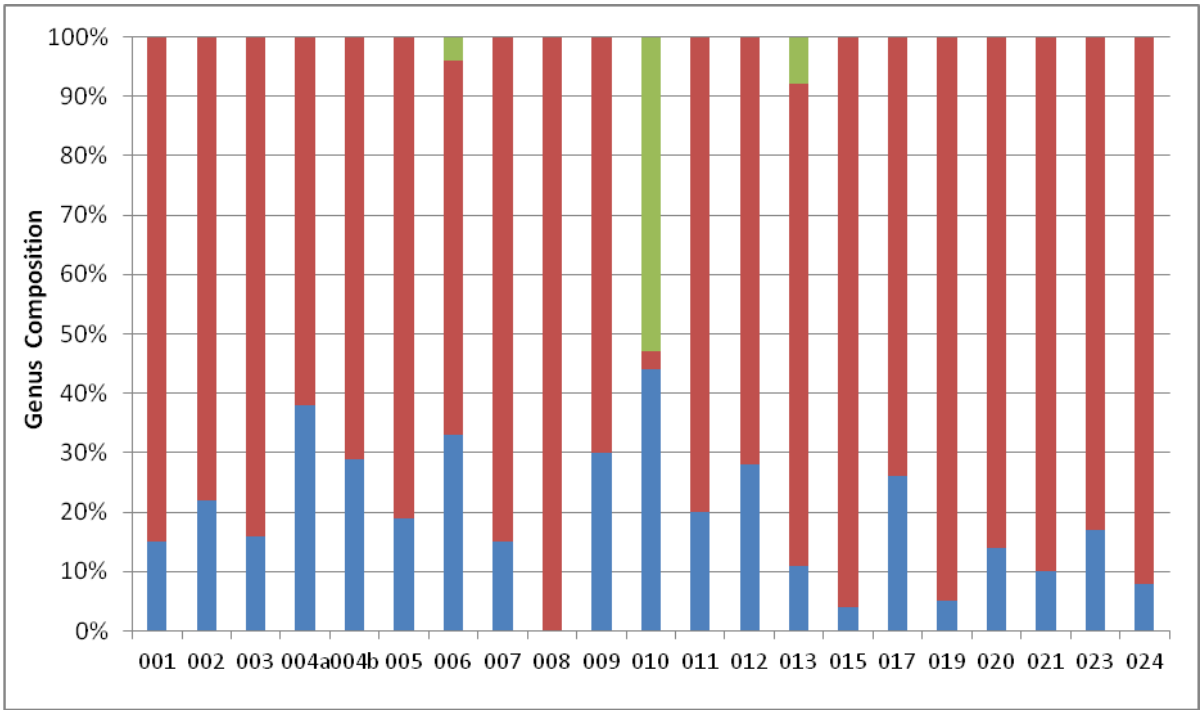

Figure 12: Percentage genus composition of trichostrongyle L_3 following faecal culture on samples obtained on day of IVM administration (Day 0). Identification was performed using morphological keys (Hansen and Shivnani, 1956; M.A.F.F., 1986) on 100 randomly selected L_3 from all samples from each farm. Blue, red and green bars indicate the presence of *Ostertagia* spp., *Cooperia* spp., and *Trichostrongylus* spp., respectively.

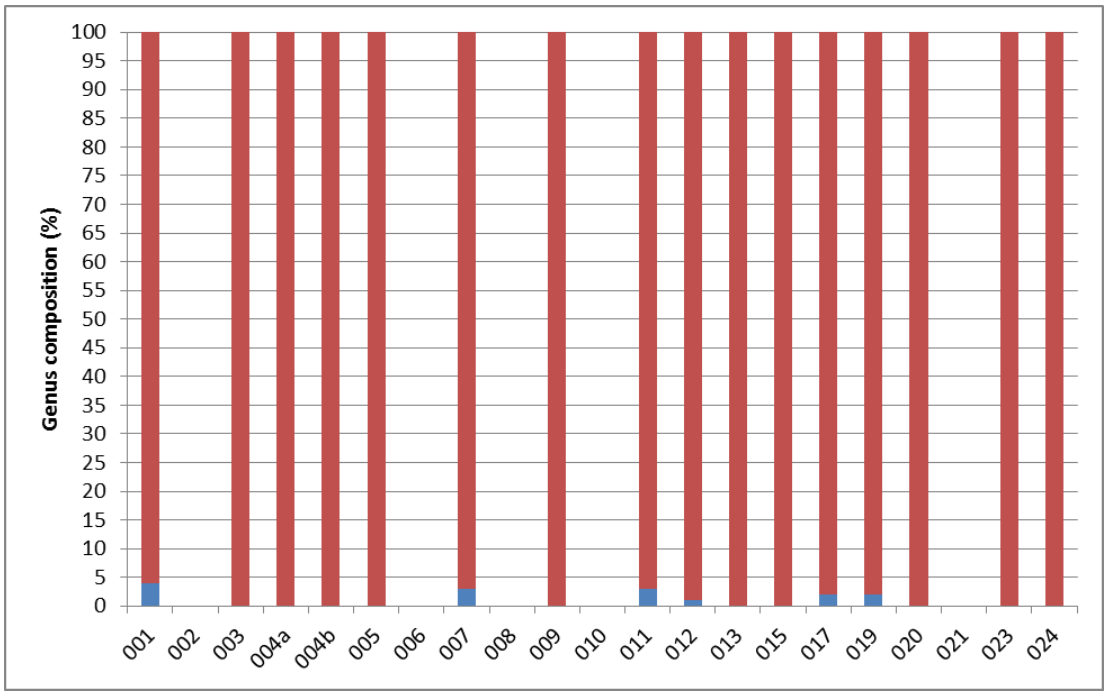

Figure 13: Percentage genus composition of trichostrongyle L_3 following faecal culture on samples 14 days following IVM administration. Identification was performed using morphological keys (Hansen and Shivnani, 1956; M.A.F.F., 1986) on 100 randomly selected L_3 from all samples from each farm. Blue and red bars indicate the presence of *Ostertagia* spp. and *Cooperia* spp., respectively. The absence of bars for Farms 006, 008, 010 and 021 indicate that no L_3 were obtained following IVM administration. Culture of samples following IVM administration from Farm 002 was not performed.

3.3.5 The impact of using different methodologies in analysing FEC and FECRT data

3.3.5.1 The influence of using 'raw' or 'combined' FEC data on FECR calculation

All faecal samples were analysed in duplicate for FEC, resulting in the production of two FEC results for each sample. WAAVP guidelines (Coles et al., 1992) do not currently account for having more than one count per sample and, as such, there may

be variation in the way data is analysed between studies. To examine this, arithmetic means (AM) of percentage reduction in FEC for each farm were calculated using both "raw" data (i.e. using both FEC results from each sample) and "combined" data (i.e. an arithmetic mean produced from combining the results of each individual sample). The results from this analysis are presented in Table 13. For samples from every farm, the classifications are the same for both "raw" and "combined" data. Production of a "combined" mean results in larger 95% CI compared to the use of "raw" data; however these smaller CI are a consequence of treating each FEC value as a separate animal, and as such, artificially inflates the dataset. In order to control this inflation, the "combined" FEC data was used for subsequent comparisons with geometric means and parametric bootstrapping.

Table 13: Table of mean percentage efficacy values generated using "raw" or "combined" FEC data to generate the arithmetic mean (AM) for each treatment population. The 95% confidence intervals are displayed in brackets. Green boxes indicate 'IVM sensitive' populations; orange boxes indicate 'IVM resistant' populations.

Farm ID	N	AM ("combined") (95% CI) [WAAVP]	AM ("raw") (95% CI)
001	6	72 (15 – 91)	72 (40 – 87)
002	10	100	100
003	16	84 (72 – 90)	84 (77 – 89)
004a	10	87 (69 – 95)	87 (77 – 93)
004b	10	65 (0 – 91)	65 (12 – 87)
005	15	78 (53 – 90)	78 (63 – 87)
006	14	100	100
007	12	74 (0 – 93)	76 (33 – 92)
008	10	100	100
009	15	98 (95 – 99)	98 (96 – 99)
010	15	100	100
011	15	66 (45 – 79)	66 (52 – 76)
012	15	88 (74 – 95)	89 (78 – 94)
013	12	51 (0 – 77)	51 (17 – 72)
015	14	67 (0 – 92)	67 (12 – 88)
017	13	97 (93 – 99)	97 (95 – 99)
019	6	67 (0 – 91)	67 (17 – 87)
020	20	97 (92 – 99)	98 (95 – 99)
021	11	100	100
023	12	0 (0 – 63)	0 (0 – 49)
024	9	86 (73 – 92)	86 (78 – 91)

3.3.5.2 The influence of generating geometric versus arithmetic means of FEC data to calculate efficacy

The use of arithmetic means has been considered to over-estimate the prevalence of anthelmintic resistance (Dash et al., 1988) and that for calculation of FECR values, the generation of a geometric mean (GM) is more appropriate. This is because the GM is based on the median FECR value which takes into account the variation between animals and reduces the effect of a few extremely high FEC values (Presidente, 1985; Dash et al., 1988; Vercruysse et al., 2001). The geometric mean also addresses the range of values within a sampling frame, and allows equal weight to be given to samples from a small range (i.e. 1 – 50) and those from a larger range (i.e. 1 – 500). The data is transformed to resemble a normal distribution, which is not always successful and can often increase error rates in subsequent analyses (Torgerson et al., 2005). However, the GM provides an estimate of the FEC of the "average" calf within a sample group, and as such, can be useful. An arithmetic mean is easier to calculate mathematically and is more often used so is more commonly the comparator across studies (Dash et al., 1988). Moreover, the arithmetic mean is not biased by the transformations applied to the GM (Fulford, 1994) and so is likely a better representative of egg output and predictions of future larval infections (Dash et al., 1988). In view of these opposing opinions, a comparison was conducted to compare FECR values generated by calculating geometric *versus* arithmetic means of the FEC data. The results from the geometric mean analysis are compared with the arithmetic mean analysis in Table 14. Efficacy values generated using AM indicated that 14 of the tested populations were IVM

'resistant'; whilst the values generated using GM indicated that one of these populations (Farm 020) was IVM 'sensitive'. GM analysis of samples from Farm 017 indicated IVM resistant nematodes were suspected, indicated by the lower 95% CI of less than 90%.

Table 14: Table of FECR values generated using arithmetic (AM) and geometric means (GM), with 95% confidence intervals displayed in brackets. 'N' represents the number of samples submitted for each population. Green, orange and blue boxes indicate IVM sensitive, resistant and suspected resistant populations, respectively.

Farm ID	N	AM (95% CI) [WAAVP]	GM FECR (95% CI)
001	6	72 (15 – 91)	63 (0 – 90)
002	10	100	100
003	16	84 (72 – 90)	81 (69 – 89)
004a	10	87 (69 – 95)	81 (52 – 93)
004b	10	65 (0 – 91)	49 (0 – 86)
005	15	78 (53 – 90)	71 (36 – 87)
006	14	100	100
007	12	74 (0 – 93)	52 (0 – 92)
008	10	100	100
009	15	98 (95 – 99)	97 (95 – 98)
010	15	100	100
011	15	66 (45 – 79)	56 (23 – 75)
012	15	88 (74 – 95)	77 (12 – 94)
013	12	51 (0 – 77)	42 (0 – 74)
015	14	67 (0 – 92)	57 (0 – 89)
017	13	97 (93 – 99)	95 (84 – 99)
019	6	67 (0 – 91)	52 (0 – 89)
020	20	97 (92 – 99)	86 (0 – 100)
021	11	100	100
023	12	0 (0 – 63)	0 (0 – 54)
024	9	86 (73 – 92)	79 (56 – 90)

3.3.5.3 The influence of parametric bootstrapping analysis on FECR calculation

The use of arithmetic and geometric means for generating FECR values has been said to fail to account for the distribution of nematode eggs within well-mixed faecal samples (Torgerson et al., 2012). If a sample is correctly mixed prior to FEC analysis, the nematode eggs are assumed to follow a Poisson distribution (Torgerson et al., 2012), as previously indicated in the enumeration of yeast cells in a haemocytometer (Student, 1907). It is known that variability within faecal samples will often result in different EPG values being generated when duplicate counts from a single sample are analysed (Vidyashankar et al., 2007), particularly when a relatively insensitive FEC method is used. To address this variability, it has been suggested that bootstrapping the data by repeated re-sampling from a Poisson distribution (for example, 10,000 times) will decrease diagnostic error rate and provide a more appropriate measure of variance between samples (Torgerson et al., 2012). Therefore, the FEC data generated from all farms before and after treatment with IVM, using the mean FEC value for each animal, was subjected to parametric bootstrapping (Torgerson et al., 2012) to produce an efficacy value. The parametric bootstrapping results are displayed in Table 15 and Figure 15. The arithmetic mean WAAVP calculated-FECR values and bootstrapping results correlate well, with classifications for all farms matching. Graphical representation of the bootstrapping analysis is shown in Figure 15. The greater the spread of the data, as indicated by the pale blue bars, the more variable the FECR values were. With Farm 020, the FECR values were closer together in comparison to other farms, indicating that the

frequency of FECR values are similar and in the region of 95% efficacy. Arithmetic mean (represented by the blue line) is the most frequently occurring FECR value, as calculated by Coles et al., (1992). As this value occurs in the middle of the pale blue bars (generated by parametric bootstrapping analysis), this serves as confirmation that the AM analysis (in combination with CF FEC method) provides an appropriate representation of the data set. Bootstrap confidence intervals were not available when there was a 100% reduction

Table 15: Parametric bootstrapping results compared to AM FECRT values. 'N' represents the number of samples submitted from each farm. Green and orange boxes indicate IVM sensitive and resistant populations, respectively.

Farm ID	N	AM FECR (95% CI) [WAAVP]	Bootstrap FECR (95% CI)
001	6	72 (15 – 91)	72 (50 – 84)
002	10	100	100
003	16	84 (72 – 90)	83 (56 – 95)
004a	10	87 (69 – 95)	87 (75 – 94)
004b	10	65 (0 – 91)	65 (8 – 88)
005	15	78 (53 – 90)	77 (43 – 93)
006	14	100	100
007	12	74 (0 – 93)	75 (0 – 100)
008	10	100	100
009	15	98 (95 – 99)	97 (93 – 100)
010	15	100	100
011	15	66 (45 – 79)	66 (45 – 78)
012	15	88 (74 – 95)	86 (72 – 97)
013	12	51 (0 – 77)	51 (30 – 64)
015	14	67 (0 – 92)	67 (27 – 85)
017	13	97 (93 – 99)	97 (91 – 100)
019	6	67 (0 – 91)	67 (38 – 82)
020	20	97 (92 – 99)	97 (88 – 100)
021	11	100	100
023	12	0 (0 – 63)	0 (0 – 32)
024	9	86 (73 – 92)	85 (75 – 91)

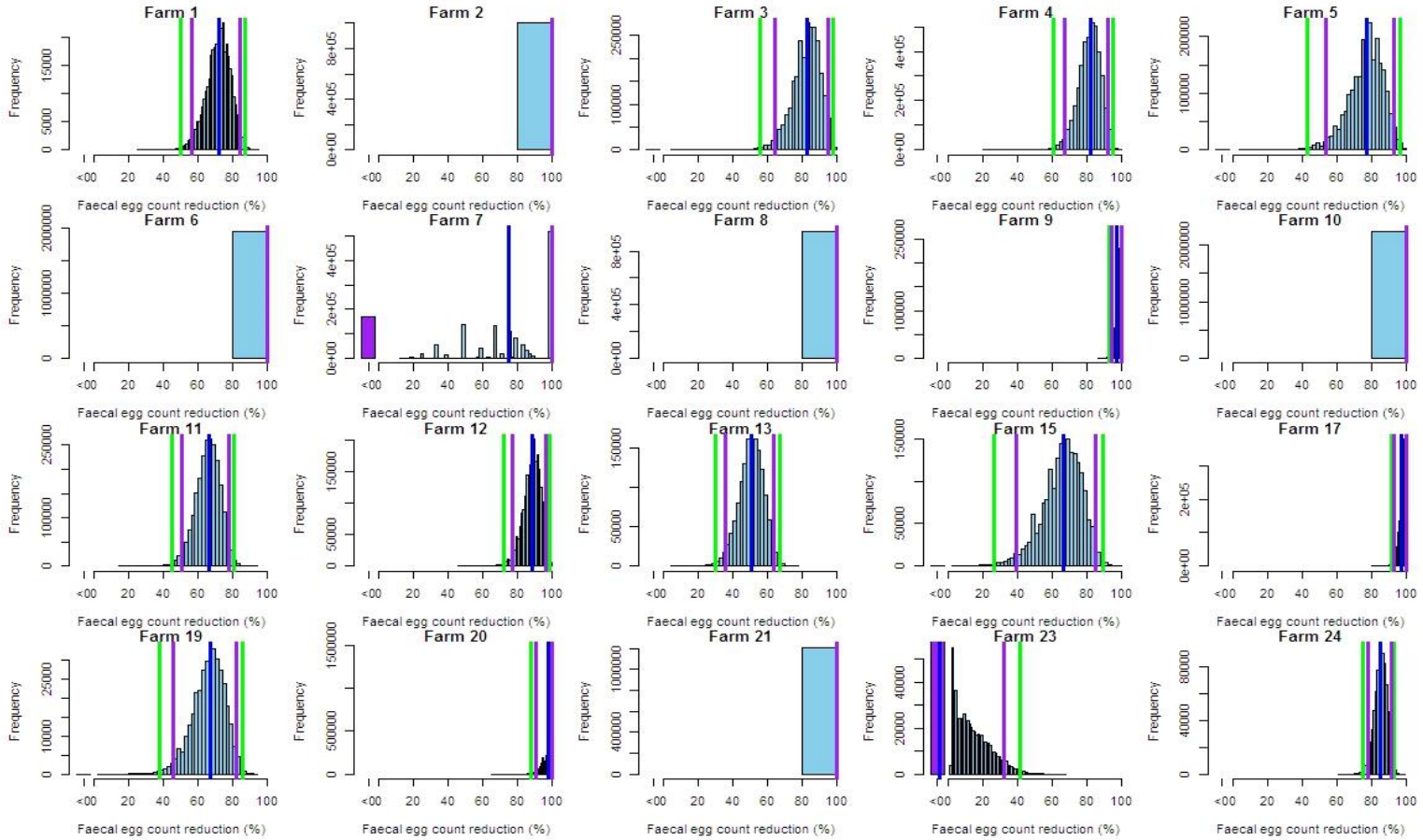

Figure 14: Graphical representation of the parametric bootstrapping analysis of IVM FECR utilising the centrifugal flotation FEC dataset for each farm. Dark blue bars represent the mean FECR as calculated by the standard WAAVP method, with the light blue bars representing the spread of FECR values after 10,000 resamplings. The purple and green lines indicate the 95 and 99% confidence intervals, with the purple boxes indicating where efficacy was less than 0%, (i.e. FEC increased following IVM administration).

3.3.5.4 Summary of FECRT classifications based on enumerating trichostrongyle nematode eggs using the CF method

A summary of the IVM sensitivity classifications produced by each type of FECR analysis is shown in Table 16. Incongruity between methods was observed for data derived from Farm 020, where a call for IVM resistance was made using the geometric mean methodology and IVM sensitivity with WAAVP arithmetic mean and parametric bootstrapping analyses. Data derived from samples from Farm 017 resulted in a classification of suspected IVM resistance with geometric mean analysis and IVM sensitivity with AM and parametric bootstrapping analyses.

Table 16: Summary table of the IVM sensitivity classifications generated using different types of analysis: WAAVP arithmetic means (AM), geometric means (GM) and parametric bootstrapping. The number in the square brackets indicates the value added to each FEC prior to GM analysis

Farm ID	N	AM FECR [WAAVP]	GM FECR [+1]	Parametric Bootstrap
001	6	R	R	R
002	10	S	S	S
003	16	R	R	R
004a	10	R	R	R
004b	10	R	R	R
005	15	R	R	R
006	14	S	S	S
007	12	R	R	R
008	10	S	S	S
009	15	S	S	S
010	15	S	S	S
011	15	R	R	R
012	15	R	R	R
013	12	R	R	R
015	14	R	R	R
017	13	S	S?	S
019	6	R	R	R
020	20	S	R	S
021	11	S	S	S
023	12	R	R	R
024	9	R	R	R

3.3.6 Impact of FECRT analysis with McMaster FEC method

It has been recommended that for the examination of cattle FEC samples, a sensitive method is employed (Coles et al., 2006). Whilst the CF method employed in the previous section is sensitive up to 1 EPG, the McMaster technique (Gordon and Whitlock, 1939) is the current "gold standard" and is used widely in practice and so facilitates direct comparison between published studies (Sutherland and Leathwick, 2011). As the CF FEC technique produced similar results in the comparison of statistical analyses, it was decided to investigate the effect of the less sensitive McMaster technique on FECRT analysis, as FECR precision is known to decrease with a less sensitive FEC method (Levecke et al., 2011). To this effect, FECRT samples from Farms 005 to 024 were also analysed in duplicate using the McMaster technique sensitive to 50 EPG (Gordon and Whitlock, 1939).

From Figure 16 it can be seen that the FEC taken on day of treatment were low, with an average FEC of 65 EPG (± 6 SEM). At a farm level, mean FEC ranged from 6 EPG (Farm 007) to 195 EPG (Farm 024). As seen with the CF method, FEC were seen to differ between farms and with samples from the same farm, for example, FEC values for Farm 011 range from 0 to 550 EPG.

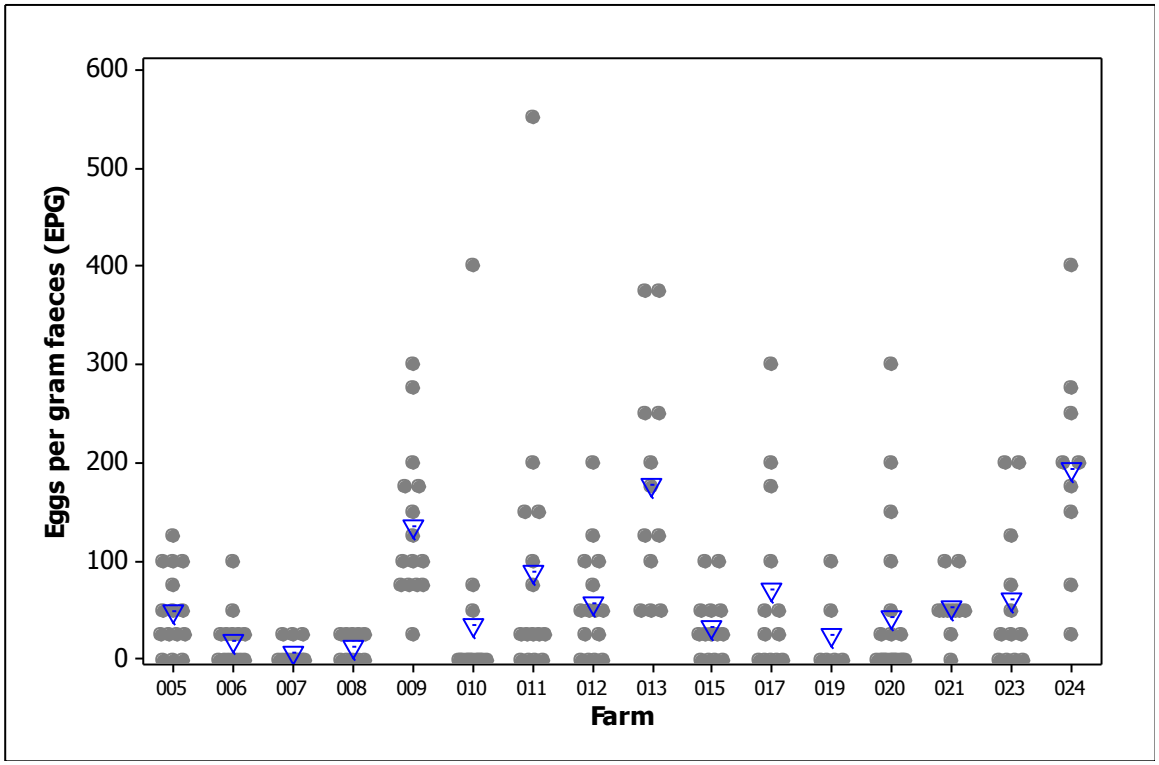

Figure 15: Trichostrongyle FEC values per gram of faeces taken on day of treatment with IVM, for each farm tested. The grey markers indicate FEC of each individual and the blue triangles indicate the mean EPG for each farm. All samples were analysed in duplicate using the McMaster FEC method

Results from samples submitted 14 days after IVM treatment are displayed in Figure 17. Zero eggs were observed in samples from Farms 005, 006, 007, 008, 010 and 021, with the mean FEC of 15 EPG (± 3 SEM) across all post-treatment samples. At farm level, the highest mean FEC was observed with samples from Farm 013 with a mean of 75 EPG (± 23 SEM). The largest range between post-treatment FEC was seen with Farm 023, with FEC ranging from zero to 275 EPG.

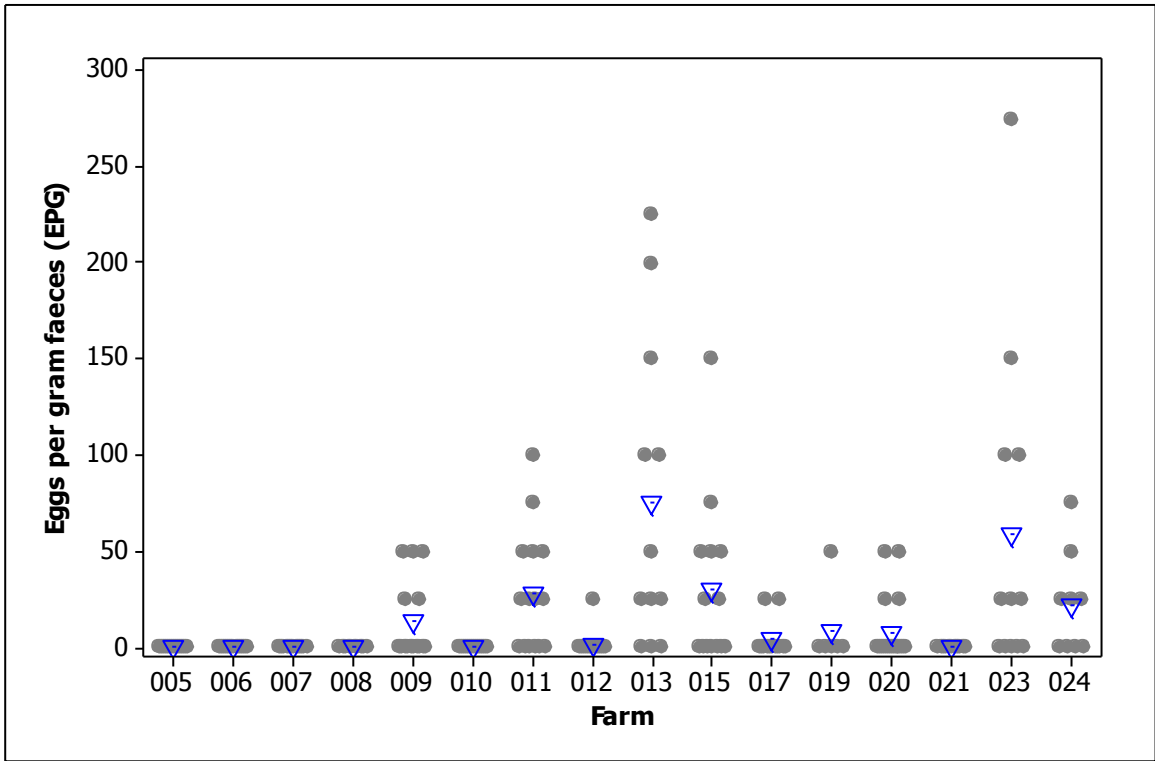

Figure 16: Trichostrongyle FEC values per gram of faeces taken 14 days following treatment with IVM, for each farm tested. The grey markers indicate FEC of each individual and the blue triangles indicate the mean EPG for each farm. All samples were analysed in duplicate using the McMaster FEC method

The arithmetic mean reduction in FEC was calculated using the formula described in Section 3.2.3.2 and the results displayed in Figure 18. Based on the WAAVP criteria described previously, data from eight farms indicated the presence of IVM resistant nematodes. Data from six farms indicated IVM-sensitivity; i.e. FECR values in excess of 95% and lower 95% CI in excess of 90%. The results from the remaining two farms indicated that IVM-resistance was suspected, as FECR values were 97% and 95%, but with lower 95% CI of 76% and 75%, on Farms 012 and 017, respectively.

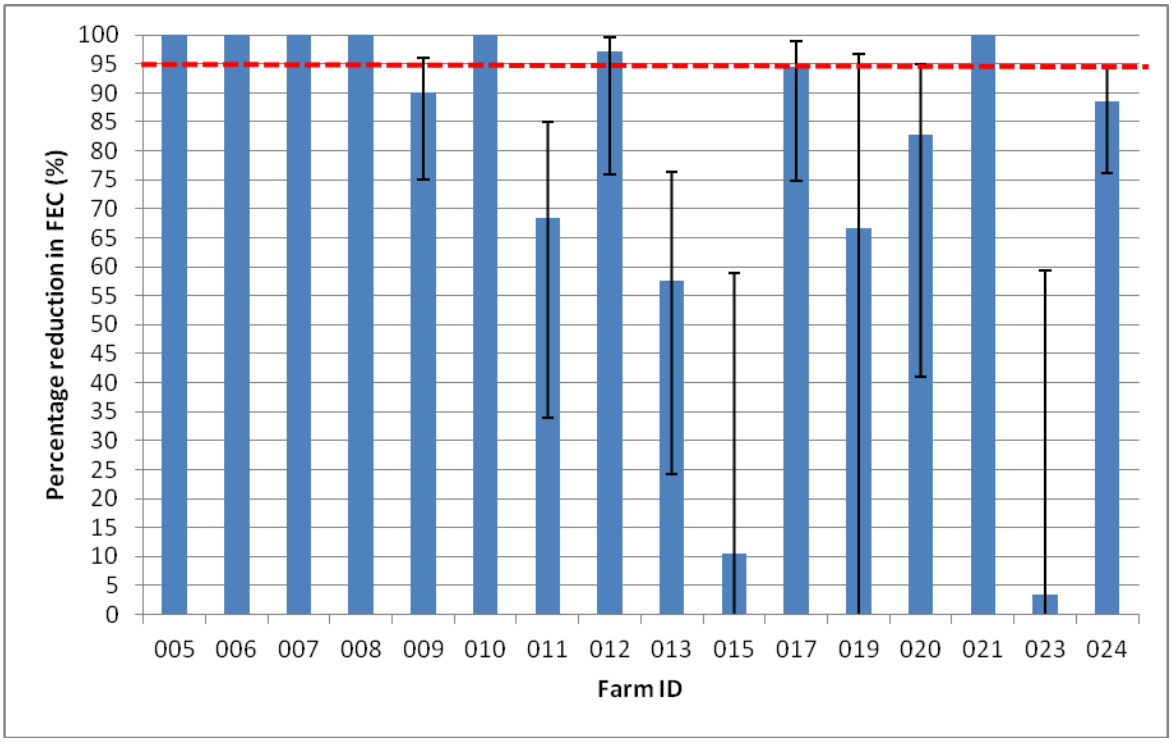

Figure 17: IVM FECRT results as assessed using the McMaster method (Gordon and Whitlock, 1939). Each bar represents percentage reduction in FEC per farm, 14 days after IVM treatment. Error bars represent the 95% confidence intervals. The red dashed line indicates the threshold for resistance as determined by WAAVP (Coles et al., 1992).

3.3.6.1 The influence of using 'raw' or 'combined' FEC data on FECR calculation using McMaster FEC method

As described in Section 3.4.1, there are no published guidelines on the use of duplicate FECs from a sample and so the effect of using "raw" or "combined" data was examined and the results are presented in Table 17. The data indicated that seven farms showed signs that the nematodes were IVM-sensitive, four in agreement with the CF FEC method analysis (Table 13; Farms 006, 008, 010, 021). Data from farms 012 and 017 indicated a "suspected resistant" population, with FECR values of 97% and 95%, respectively, but with lower CI of below 90%. With the CF methodology, Farm 012 was classified as being an IVM resistant

nematode population in all analyses, whereas for Farm 017, resistance was suspected with GM analysis and the population considered sensitive with the AM and bootstrap analysis.

Table 17: Table of FECR data derived using the McMaster FEC method, based on using "raw" or "combined" data to generate arithmetic means (AM). 95% confidence intervals are displayed in brackets. Green boxes indicate "IVM sensitive" populations, orange boxes indicate "IVM resistant" populations and blue boxes indicate "suspected IVM resistance" populations.

Farm ID	N	AM ("combined") (95% CI)	AM ("raw") (95% CI)
005	15	100	100
006	14	100	100
007	12	100	100
008	10	100	100
009	15	90 (76 – 96)	90 (75 – 96)
010	15	100	100
011	15	69 (13 – 89)	69 (34 – 85)
012	15	97 (76 – 100)	97 (76 – 100)
013	12	58 (11 – 80)	58 (24 – 76)
015	14	11 (0 – 65)	11 (0 – 59)
017	13	95 (74 – 99)	95 (75 – 99)
019	6	67 (0 – 97)	67 (0 – 97)
020	20	83 (36 – 95)	83 (41 – 95)
021	11	100	100
023	12	3 (0 – 69)	3 (0 – 59)
024	9	89 (72 – 95)	89 (76 – 95)

To ensure comparable results with the CF FEC method, it was decided to use the "combined" FEC data for the comparisons with geometric means and parametric bootstrapping analysis.

3.3.6.2 The influence of generating geometric *versus* arithmetic means of McMaster FEC data to calculate efficacy

The results from the GM analysis are presented in Table 18 and are predominantly in agreement, with the dataset which used AM in the analysis. Two exceptions were observed: one with Farm 012, where the addition of 1 prior to GM analysis produces an IVM-resistant result. In contrast, a 'suspected resistance' result was generated with AM analysis and GM when 25 was added prior to analysis. The second exception was observed with Farm 017, where the GM analyses indicate IVM resistance, whereas the AM analysis suggests 'suspected resistance'. Overall, the McMaster GM (with the addition of 25) appears to compare more favourably to the results generated with the McMaster AM data, than the McMaster GM results with a value of one added.

Table 18: Table of FECR values obtained using McMaster method to generate arithmetic (AM) and geometric means (GM), with 95% confidence intervals displayed in brackets. 'N' represents the number of samples submitted from each farm. Green boxes indicate IVM sensitive farms, orange boxes indicate IVM resistant farms and blue boxes indicate suspected IVM resistant farms

Farm ID	N	AM FECR (95% CI) [WAAVP]	GM FECR (95% CI) [+1]	GM FECR (95% CI) [+25]
005	15	100	100	100
006	14	100	100	100
007	12	100	100	100
008	10	100	100	100
009	15	90 (76 – 96)	88 (73 – 95)	92 (73 – 98)
010	15	100	100	100
011	15	69 (13 – 89)	0 (0 – 96)	55 (0 – 93)
012	15	97 (76 – 100)	92 (43 – 99)	97 (42 – 100)
013	12	58 (11 – 80)	47 (0 – 76)	69 (10 – 89)
015	14	11 (0 – 65)	0 (0 – 52)	26 (0 – 82)
017	13	95 (74 – 99)	72 (0 – 100)	93 (20 – 99)
019	6	67 (0 – 97)	0 (0 – 100)	65 (0 – 99)
020	20	83 (36 – 95)	0 (0 – 100)	75 (0 – 98)
021	11	100	100	100
023	12	3 (0 – 69)	0 (0 – 81)	14 (0 – 89)
024	9	89 (72 – 95)	86 (65 – 94)	90 (67 – 97)

3.3.6.3 The effect of parametric bootstrapping on FECR calculation with McMaster method

As explained in Section 3.4.3, there is a need to account for the FEC variability seen within samples and so the McMaster FEC data were treated in the same way, with FEC values resampled 10,000 times from a Poisson distribution (Torgerson et al., 2012) . The FECRT values are presented in Table 19 and in Figure 19. The McMaster data presented here shows greater variability in FECR frequencies compared to the CF results, made apparent by the wider spread of the data in Figure 19 compared to that in Figure 15. From Table 19, the classifications using the AM method and bootstrapping, are the same for 14 of the 16 farms tested. The exceptions are seen with Farms 012 and 017. With Farm 012, where the FECR values are the same (97%) but a lower 95% CI of 76% gives a call for suspected IVM resistance with the AM, whereas the bootstrapping method generates a call for sensitivity based on a lower 95% CI of 91%. With Farm 017, the AM method produces a call for suspected IVM sensitivity, due to a FECR of 95% and lower 95% CI of 74%, however this changes to IVM resistant when bootstrap analysis is conducted, producing a FECR of 94% and a lower 95% CI of 88%. Bootstrap confidence intervals were not available when there was a 100% reduction

Table 19: Parametric bootstrapping results for McMaster method, compared to FECR values generated with AM. 'N' represents the number of samples submitted from each farm. Green boxes indicate IVM sensitive farms, orange boxes indicate IVM resistant farms and blue boxes indicate suspected IVM resistant farms

Farm ID	N	AM FECR (95% CI) [WAAVP]	Bootstrap FECR (95% CI)
005	15	100	100
006	14	100	100
007	12	100	100
008	10	100	100
009	15	90 (76 – 96)	90 (84 – 95)
010	15	100	100
011	15	69 (13 – 89)	69 (54 – 81)
012	15	97 (76 – 100)	97 (91 – 100)
013	12	58 (11 – 80)	58 (45 – 68)
015	14	11 (0 – 65)	12 (0 – 47)
017	13	95 (74 – 99)	94 (88 – 99)
019	6	67 (0 – 97)	68 (33 – 89)
020	20	83 (36 – 95)	82 (65 – 94)
021	11	100	100
023	12	3 (0 – 69)	3 (0 – 33)
024	9	89 (72 – 95)	88 (83 – 93)

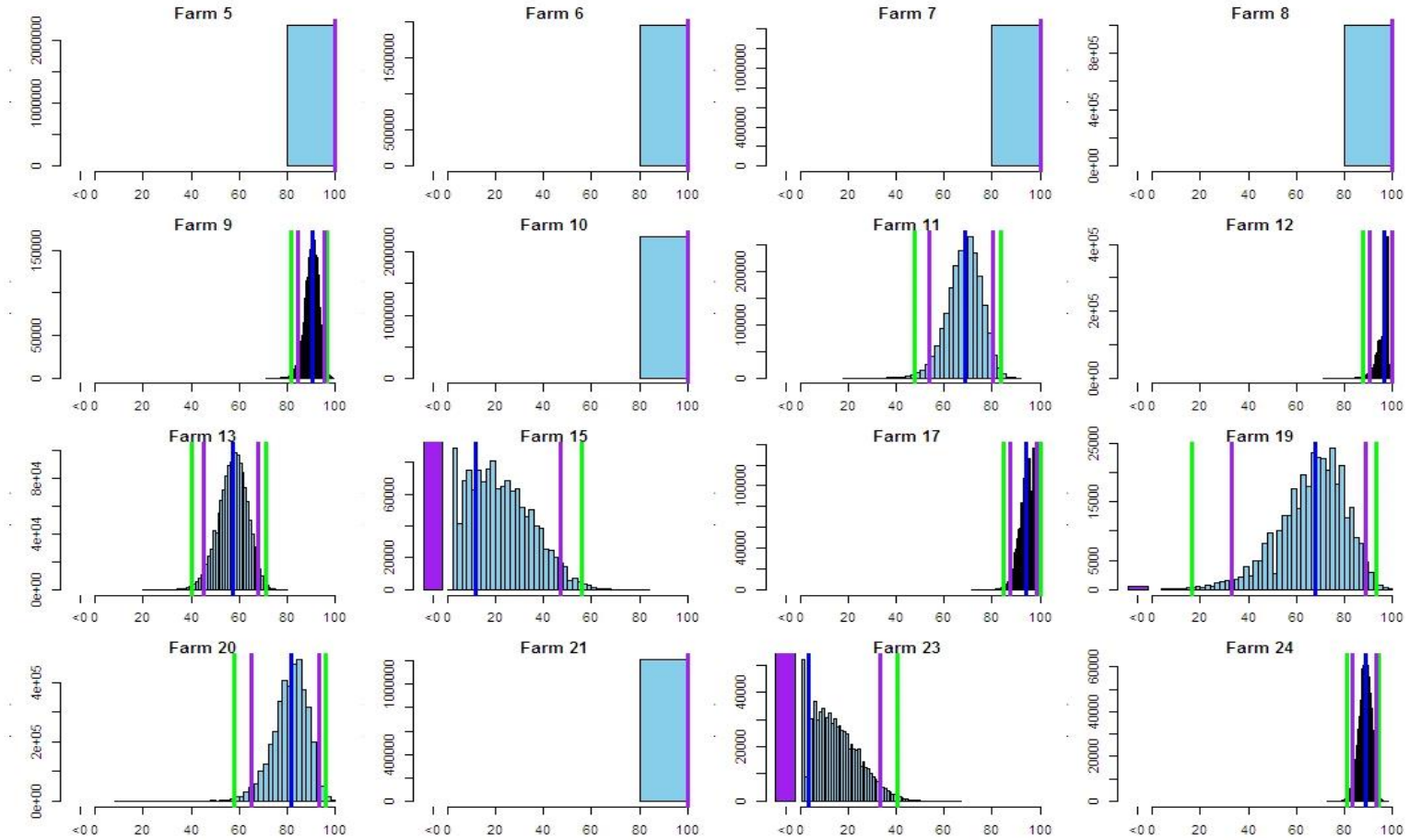

Figure 18: Graphical representation of the parametric bootstrapping using McMaster data for each farm tested. Dark blue bars represent the mean FECR as calculated by the standard WAAVP AM method, with the light blue bars representing the spread of FECR values after 10,000 resamplings. The purple and green lines indicate the 95 and 99% confidence intervals purple boxes indicating where efficacy was less than 0%, (i.e. FEC increased following IVM administration).

3.3.6.4 Summary of FECRT classifications based on enumerating trichostrongyle nematode eggs using the McMaster method

A summary of the classifications produced for each FECR analysis, for the McMaster FEC data is shown in Table 20. Disagreements in classification are seen with Farms 012 and 017. Both GM methods are in agreement, but with Farm 012, the AM method indicates suspected IVM resistance, and bootstrapping indicated IVM sensitivity. With Farm 017, both GM methods call IVM resistance, but this is only suspected IVM resistance with AM and bootstrapping. Overall, as seen with the CF analysis above, AM analysis with the McMaster FEC method appears to be as robust as the more mathematically involved processes of the GM and parametric bootstrapping methods.

Table 20: Summary table of the IVM resistance classifications generated using different analysis methodologies: arithmetic means (AM), geometric means (GM) and parametric bootstrapping. The numbers in the square brackets indicate the value added to each FEC prior to GM analysis

Farm ID	N	AM FECR [WAAVP]	GM FECR [+1]	GM FECR [+25]	Parametric Bootstrap
005	15	S	S	S	S
006	14	S	S	S	S
007	12	S	S	S	S
008	10	S	S	S	S
009	15	R	R	R	R
010	15	S	S	S	S
011	15	R	R	R	R
012	15	S?	R	R	S
013	12	R	R	R	R
015	14	R	R	R	R
017	13	S?	R	R	S?
019	6	R	R	R	R
020	20	R	R	R	R
021	11	S	S	S	S
023	12	R	R	R	R
024	9	R	R	R	R

From the samples where the McMaster and CF techniques were both conducted, five classifications from a total of 16 did not agree in all analyses, namely Farms 005, 007, 009, 012, 017 and 020. With Farms 005 and 007, both were classified as resistant with the CF method and as sensitive with the McMaster analyses, whereas the inverse was found with

Farm 009. With the remaining farms, differences in classification were not restricted to FEC method, but differed dependent on the use of GM analysis.

3.3.7. Comparison of CF and McMaster percentage efficacies

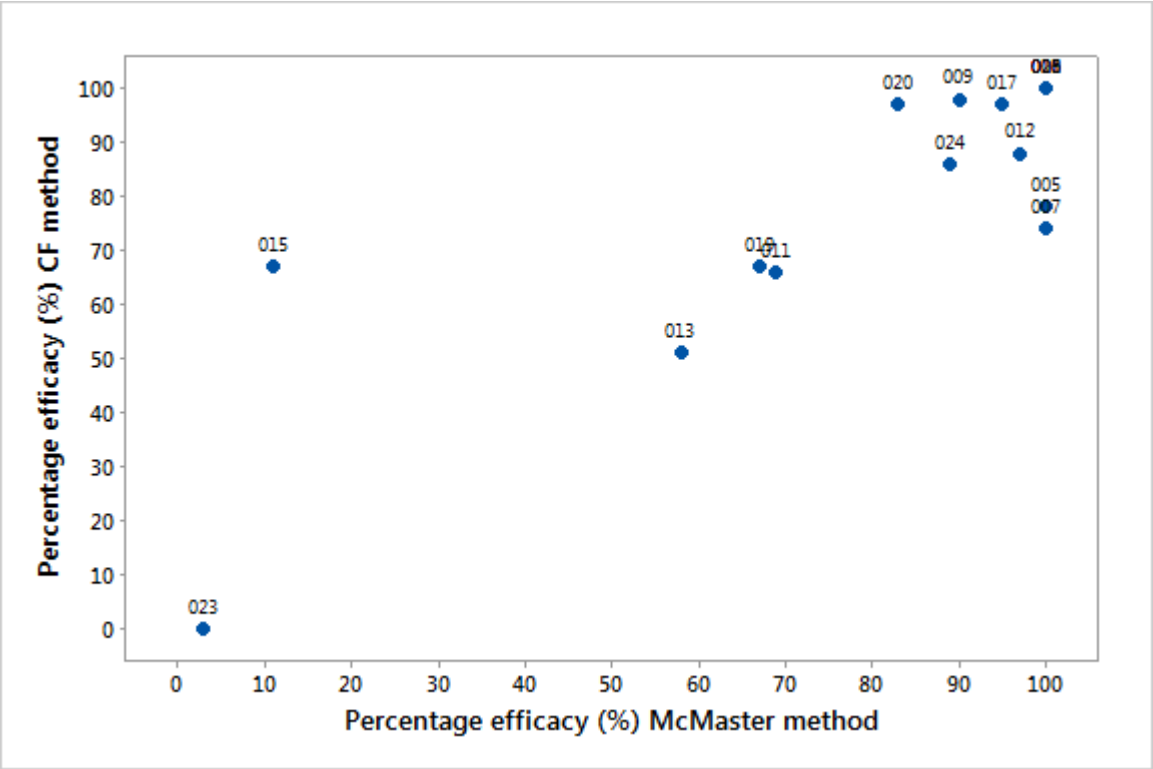

Figure 19: Comparison of percentage efficacies for farms where FEC were conducted using both CF and McMaster methodologies. Data labels indicate farm name.

Figure 19 illustrates a graphical comparison between percentage efficacies calculated for the farms where both FEC methodologies were employed.

3.3.8. Analysis of risk factors for IVM resistance

From the univariable odds ratio (OR) analysis, there were two areas where a statistically significant difference was found. First, on farms where IVM resistant nematodes were present, farmers were significantly less likely to seek advice on parasite control from either

other farmers or pharmaceutical representatives (ORs < 0.07, 95% confidence intervals (CI) 0.01 – 0.8). Second, on farms where IVM resistance was present, farmers were significantly more likely to isolate newly acquired cattle following quarantine (OR 18, 95% CI: 1.3 – 255). Fewer stock were farmed where IVM resistance was identified (mean of 52 animals per age group) compared to farms with IVM sensitive populations (67 animals per age group), but adult cattle were managed at slightly higher stocking densities (3 – 5 per hectare) on four farms. The number of anthelmintic treatments administered each year was also similar; with an average of 1.6 treatments administered on farms where IVM resistant nematodes were identified compared to an average of 1.9 treatments administered per year on farms where IVM sensitive nematodes were identified. ML treatments were used by all farmers and administered as a pour-on application. None of the farmers in either group believed their anthelmintic efficacy was lower compared to previous years. One area where the groups (i.e. farms where IVM resistant *vs.* IVM sensitive nematodes were identified) differed in management was in the treatment of adult cows. On five of the farms where IVM-resistant nematodes were detected, the farmers treated adult cattle at housing, compared to the group in which where IVM-sensitive nematodes were detected where only one farmer treated cows at this time. In addition, on six of the 13 farms where IVM resistance was detected, the farmers treated all of their adult cows. This is in contrast to treating them on a selective basis, which was conducted on one of the farms where IVM sensitive nematodes were identified. Treatment practices for younger cattle (stirks and calves) were similar in both groups, with all animals receiving anthelmintic treatments on average twice a year.

3.4 Discussion

As a result of the increase in the number of reports of AR in cattle nematodes worldwide, there is a need for robust tests to improve detection of resistance and allow comparisons to be conducted between studies (Sutherland and Leathwick, 2011). Here, IVM efficacy was assessed on 21 sets of faecal samples from 260 FSG calves on 20 farms, with IVM resistant *Cooperia* spp. identified on 13 farms. FEC recorded prior to IVM treatment were relatively low, with 90% of samples below 150 EPG, with a mean of 54 EPG across all 260 calves tested. Highly aggregated k values (i.e. $k < 1$) in the FEC generated from these samples indicate that the distribution of nematode eggs in these cattle fits well with the negative binomial distribution, which has been previously shown to be an appropriate distribution for macroparasites (Shaw and Dobson, 1995) and widely believed to be representative of all gastrointestinal nematode infections (Shaw et al., 1998; Morrill and Forbes, 2012). These FEC results correlate well with FEC levels observed previously in naturally infected FSG calves at this time of year, albeit calculated using differing FEC methodologies (Cornwall et al., 1971; Lyons et al., 1995; Ellis et al., 2011).

In the UK, FEC research has predominately focussed on experimental infections for anthelmintic registration purposes; however work recently conducted on an organic cattle farm in Scotland found low FEC (<50 EPG) in FSG calves throughout the winter housing period (Ellis et al., 2011). An earlier study also identified that, between farms, mean FEC can differ, and the time at which FEC peak in the season can differ, dependent on pasture management and climatic factors (Cornwall et al., 1971). This has also been observed in other temperate regions, such as parts of the USA (Lyons et al., 1995) and mainland Europe (Kerboeuf et al., 1996; Poot et al.,

1997; Jäger et al., 2005; El-Abdellati et al., 2010a). Other studies also reported low FEC in FSG calves, with mean FEC of 50 EPG to 100 EPG reported in France (Kerboeuf et al., 1996); the Netherlands (Poot et al., 1997) and Germany (Jäger et al., 2005). Little information was reported regarding the FEC method employed in these studies; however, a study conducted across Belgium and Germany reported a mean FEC of 90 EPG when a McMaster FEC method sensitive to 50 EPG was used (El-Abdellati et al., 2010a). The similarity of FEC distribution across the three years of sampling (2010 – 2012) here matches a previously published observation (Gasbarre et al., 1996), where the largest source of variation in FEC was found between samples from calves on the same farm. The relatively low FEC values observed here may also be attributed to the time of year when sampling was conducted, as FECs have previously been shown to be lowest at housing in the UK (Michel et al., 1970a; Bairden et al., 1985).

The current FECRT study was designed to follow the farmers' routine treatment regimes as far as possible (Chapter 2), which included treatment of FSG calves at housing after grazing over the summer months with their dams. A benefit of conducting the FECRT sampling at housing is that for collection of samples following treatment, calves do not have to be gathered in from grazing thus reducing time and labour costs and increasing the willingness of participants to comply with sampling schedules. Consequently, the results provide valuable data regarding FEC levels when this anthelmintic treatment is normally administered. It is important to note that FECs are not particularly informative of total nematode infection levels, especially as both *Ostertagia* spp. and *Cooperia* spp. larvae are known to inhibit in

the gastrointestinal tract during winter months (Armour et al., 1969a; Michel et al., 1970a). The re-emergence of inhibited *Ostertagia* spp. larvae in spring, can lead to Type II ostertagiosis, which can prove fatal (Martin et al., 1957; Armour, 1970; Armour and Duncan, 1987) and this is one of the main reasons that a treatment at housing with an appropriate anthelmintic is recommended, i.e. to eliminate these larvae. MLs are currently the only anthelmintic class licensed in UK cattle with claims of high efficacy against inhibited *O. ostertagi* larvae (NOAH, 2014). Efficacies against inhibited *O. ostertagi* stages have been reported to be in excess of 99% when compared to untreated control animals (Egerton et al., 1981; Yazwinski et al., 1981; Benz et al., 1983; Alva-Valdes et al., 1984). In contrast, there have been conflicting reports as to the effectiveness of BZ anthelmintics, particularly fenbendazole (FBZ), against these inhibited stages (Anderson and Lord, 1979). Some studies have published efficacies in excess of 97% (Duncan et al., 1976; Lancaster and Hong, 1977; Duncan et al., 1978); however, others demonstrated efficacies in the region of only 20% (Lancaster and Hong, 1977) or no significant difference in inhibited larval burdens when treated animals were compared to untreated infected control animals (Elliott, 1977).

Based on an arithmetic mean FECR calculation as recommended by the WAAVP (Coles et al., 1992), with the CF FEC technique, 13 farms tested here showed indications of IVM resistance, based on a FECR of less than 95% and a lower 95% CI of less than 90%. Despite little published information regarding FECRT analyses in UK cattle, this finding is not wholly unexpected, given previous reports of IVM resistance from Coles and Stafford (1999), Coles et al., (2001) and reports of ML

inefficacy in calves from Sargison et al., (2009, 2010). The current study is the largest conducted in the UK to date. Within northern Europe, FECRT reporting is limited to two main studies (Demeler et al., 2009; El-Abdellati et al., 2010a). In the first, results similar to those reported here were found in samples collected from farms in Belgium (n = 8), Germany (n = 9) and Sweden (n = 5), before and after parenteral administration of IVM (Demeler et al., 2009). Samples from three farms were taken 14 days following treatment, with the remainder sampled seven days following treatment. All animals were then sampled again 21 days after treatment. Analysis at Day 7 or 14 revealed six farms with nematode FECR of less than 95%, increasing to 16 farms at 21 days following treatment (Demeler et al., 2009). Collection of samples seven days following IVM administration is not advised for FECRT analysis, as egg suppression in female nematodes following ML treatment has been reported (Presidente, 1985; McKellar et al., 1988; Coles et al., 2006). It is currently recommended to sample cattle 14 – 17 days after ML anthelmintic administration, to avoid false results (Coles et al., 2006). After 14 days, the egg suppressive effect on adult nematodes is thought to be minimal (Leathwick and Miller, 2013). In the previous EU study (Demeler et al., 2009), faecal cultures taken before and after IVM administration showed the presence of *Ostertagia* and *Cooperia* spp. larvae prior to treatment, with *Cooperia* spp. predominating following treatment in samples analysed at Day 7 and Day 21 after treatment. These were reported as presence/absence for each species as opposed to the relative proportions of species identified. As IVM is not licensed for high efficacy against immature larval stages of *Cooperia* spp. (NOAH, 2014), eggs present in samples collected 21 days after treatment may be the result of these stages reaching patency, rather than

resumption of egg laying from surviving adult nematodes (Wood et al., 1995). In a subsequent study conducted in Belgium, a FECR of less than 95% was identified after ML administration on 33 of 84 farms studied (El-Abdellati et al., 2010a). Faecal cultures conducted using samples from 20 of the farms with positive FEC results following treatment revealed that, again, *Cooperia* spp. larvae dominated the cultures, although the presence of other species (including *Ostertagia* spp.) were noted (0.5 – 2.5%; (El-Abdellati et al., 2010a).

For subsequent control programs to be optimised, differences in parasite management practices between farms where IVM resistance has been detected and those where IVM was deemed efficacious, need to be considered. From the univariable OR analysis here, on farms where IVM resistance was present, farmers were significantly more likely to isolate newly acquired cattle following quarantine. This was an unexpected finding, as it would be thought that farmers who operated an effective quarantine procedure would be less likely to have selected for drug resistant parasites. However, when the class of anthelmintic used for quarantine purposes was examined, an ML anthelmintic was preferred (see Chapter 2, Section 2.3.2.1); indicating appropriate quarantine procedures were not being followed. Fewer stock were farmed on farms where IVM resistance was identified, but adult cattle were kept at slightly higher stocking densities on four of the farms where IVM resistance was detected. The number of anthelmintic treatments administered each year was similar between farms where IVM resistance was detected and those where it was not. None of the farmers in either group believed anthelmintic efficacy to be reduced compared to previous years. One area where the groups differed in management was

in the treatment of adult cows. On farms where IVM sensitive nematodes were identified, adult cattle were administered with anthelmintics less frequently or on a more selective basis. These results indicate that selection pressure for anthelmintic resistance may be being applied through the treatment of adult cattle. Although adult cows are considered to carry low nematode burdens due to immunity, worms can be present, and due to the greater faecal volume produced, this can potentially amount to substantial contamination over the course of a grazing season (Borgsteede, 1978). In studies on anthelmintic resistance in sheep, the practice of not administering anthelmintics to adult ewes whilst they are grazing with lambs has been reported to provide refugia for some parasitic nematode species such as *Cooperia* spp., but not for others such as *Nematodirus* spp. (Leathwick et al., 2008).

The presence of predominantly *Cooperia* spp. in faecal samples following IVM administration is a finding not unique to this study (Coles et al., 1998; Coles et al., 2001). *Cooperia* spp. have previously been identified to be one of the dose-limiting species for IVM, requiring higher levels of anthelmintic to achieve efficacy compared to other nematode species (Egerton et al., 1981; Eagleson and Allerton, 1992) (Sutherland and Leathwick, 2011). *Cooperia* spp. have been reported to be more fecund than *Ostertagia* spp., and hence responsible for a high proportion of eggs counted in FEC analysis (Kloosterman, 1971). However, they are considered to be less pathogenic than *Ostertagia* spp. and so risk being considered less important (Sutherland and Leathwick, 2011). As a result, it has been suggested that parasite control practices should focus on the control of *O. ostertagi* (Malczewski et al., 1996). Nonetheless, reports have been published regarding weight loss and

diarrhoea in experimental monospecific infections with *Cooperia* spp. (Coop et al., 1979; Sutherland and Leathwick, 2011). Softening of faeces during infection has been reported (Borgsteede and Hendriks, 1979; Coop et al., 1979; Armour et al., 1987) and statistically significant differences in live weight gain were identified between calves infected with 200,000 *C. oncophora* L_3 and calves infected with 20,000 L_3 (Borgsteede and Hendriks, 1979) In one study, a mean weight gain difference of 16 kg was observed between *Cooperia* infected calves and infected animals treated effectively with an anthelmintic after 10 weeks (Armour et al., 1987). Furthermore calves infected with IVM resistant isolates have been reported to show diarrhoea and remain in poor condition compared to calves infected with an IVM sensitive isolate (Coles et al., 2001; Sargison et al., 2010). Diarrhoea, rough hair coat and unthriftiness have also been reported in calves infected with 10,000 larvae from an IVM resistant *C. oncophora* isolate; symptoms which were not observed with calves infected with an identical number of larvae from an IVM sensitive *C. oncophora* isolate (Njue and Prichard, 2004c) .

In regions where more, or larger, studies have been performed (for example, New Zealand), detection of ML resistant *Cooperia* spp. has often preceded the observation of AR in other nematode species and multi-class resistance (Sutherland and Leathwick, 2011). Sampling of a larger cohort may have revealed ML resistance in *O. ostertagi* in the current study. It should also be considered that the FECRT is only really able to define resistance in adult nematodes, and so the possibility of developing resistance, for example in the increased survival of inhibited *O.ostertagi* larvae in the abomasal mucosa after treatment would go undetected (Eysker and

Ploeger, 2000; Vercruysse and Claerebout, 2001). With this in mind, the presence of ML resistant *O. ostertagi* isolates in UK cattle cannot be discounted.

The FECRT is not a perfect test; any guidelines set in place for detection of AR require a degree of flexibility (Coles et al., 1992; van Wyk and Groeneveld, 1997). The WAAVP guidelines recommend a minimum of 10 animals per treatment group, and each animal should have a nematode FEC in excess of 100 EPG (Coles et al 2006). This figure is based on McMaster FEC methodology, usually only sensitive to 50 EPG (Gordon and Whitlock, 1939). The guidelines also stipulate that animals should not have been treated with anthelmintic in the preceding 8 to 12 weeks and that an untreated cohort of animals is sampled concurrently, to monitor any 'natural' change in FEC output not associated with treatment. The use of oral anthelmintics is encouraged, and resistance determined based on a calculation of FECR using the AM where a reduction of less than 95% is indicative of resistance, when combined with a lower 95% confidence interval of less than 90% (Coles et al., 1992). Several of these requirements are not particularly appropriate for use with cattle. First, the FEC at the time of treatment may be low as identified here; however, a sensitive FEC method can be employed (Jackson, 1974) to overcome the statistical limitations of defining resistance based on low initial FECs. The impact of using a sensitive FEC method here was the ability to detect FEC down to 1 EPG, and this led to less variability between classifications derived from the subsequent data analysis. Second, the recommendation of using oral anthelmintics is not feasible in UK cattle, as ML compounds are now only available as pour-on or injectable formulations (NOAH, 2013, VMD database). The use of an untreated control group could also prove

contentious, with all the participating farmers in this survey intending to treat all of their calves at housing. In this study, a number of measures were put in place to facilitate completion of the FECRT as far as possible in accordance with the WAAVP guidelines. Equipment for administering anthelmintic treatment and weigh tapes were provided to ensure all animals were given a dose appropriate for individual weight. Sampling was always conducted on the day of treatment and 14 days following treatment. As recommended (Coles et al., 1992) , faecal cultures were used to provide larvae that were analysed to identify any change in nematode genus/ species proportions following treatment.

As there is no standardised method for the FECRT in cattle, comparisons between studies are difficult (Sutherland and Leathwick, 2011). Whilst the McMaster FEC method has been found to be highly repeatable, with little confounding effect on variance within faecal samples (Denwood et al., 2012), it has been identified that there is a significant increase in the precision of a FECR result when a more sensitive FEC method is used (Levecke et al., 2011). Previously published work has shown that use of a FEC technique sensitive to 1 EPG is significantly more precise in the attribution of FECR classification ($P < 0.0001$) than a FEC technique sensitive to 50 EPG, and this has been attributed to the greater multiplication effect of the McMaster technique (Levecke et al., 2011). Due to the low FEC levels at treatment observed here, it is logical that a technique with a higher degree of sensitivity be employed for the FECRT. Here, greater numbers of zero FECs were observed with McMaster methodology; specifically 35% of the day of IVM administration FECs compared to only 3% detected as 0 FEC using the CF method. Variations in bovine FECs have

been largely associated to be a result of variation between individual animals sampled, rather than the variation within the faecal sample (Gasbarre et al., 1996); however, decreases in the variability of bovine and ovine FECs have been observed in the use of repeated counts (Gasbarre et al., 1996; Morgan et al., 2005). When a zinc sulphate centrifugal flotation FEC (sensitivity < 10 EPG) was used, sample variance was reduced by 20% when duplicate FEC were made instead of one count (Gasbarre et al., 1996). This was reduced further (by a total of 30%) by undertaking five repeat FECs per sample, but the additional time and effort involved in conducting analysis past two counts was deemed not to be worthwhile (Gasbarre et al., 1996). Similarly, for McMaster FEC methods, there was a substantial decrease in the predicted improvement of FEC accuracy when more than two slides (i.e. four chambers) were counted (Morgan et al., 2005). Consequently, the decision to use duplicate FEC for both CF and McMaster methods in this study is justified.

The geometric mean is considered by some to be the most appropriate analysis for skewed data (such as helminth egg distributions) as it accounts for the variation within a selection of faecal samples. In doing so, GM analysis provides a mean FEC value for a sampled cohort which is not influenced by outlying data points, such as FECs with zero values, and is close to the median value, which indicates a data point in the middle of the data set (Smothers et al., 1999). To achieve this, all data must be transformed to fit a log-normal population distribution, achieved by taking the logarithm of each FEC value (Smothers et al 1999). The subsequent GM value is then used for making a statistical inference on anthelmintic efficacy and can allow comparison between populations, with the caveat that identical transformations are

made (Presidente, 1985; Smothers et al., 1999; Vercruysse et al., 2001). The GM is influenced less by a small percentage of high values (Fulford, 1994), as present in this dataset. In contrast, the AM is thought to be more suitable for determining levels of nematode egg output from animals on an individual basis. This is because AM values are directly representative of the total egg output of the group (Dash et al., 1988), as they are not biased by transformations required for generating GM (Fulford, 1994). The AM is also simpler to calculate and is considered a more conservative estimate of parasitic isolate sensitivity to anthelmintics (Dash et al., 1988). As a result of the transformation processes, GM values are lower than AM values (Smothers et al., 1999). On a practical level, this means that when used with FECRT data, GM means may generate a lower FECRT value, and by labelling a population as resistant when it may not be when the FECRT is calculated by another method, resistance levels in nematode populations may be overestimated. Additionally, if GM FEC values are used to determine when to give an anthelmintic treatment, the low mean values generated may lead to animals not receiving a treatment when one is anticipated based on that individual's FEC (McKenna and Simpson, 1987). Based on the CF results presented here, 18 of the 21 FECRT results concurred regarding IVM sensitivity classification across all types of analyses. With the McMaster data, 14 of the 16 sets of FECR results agreed, indicating generally a good agreement between the AM and GM analyses. This is comparable to data analysed by Dash et al (1988), where the results from only three FECRTs from a total of 22 did not concur in sensitivity classification when GM+1 FECRs were compared to AM and GM+20 FECR results. Overall, from this study, FECR

analysis based on the calculation of the AM appears to be as robust as the more mathematically involved processes of the GM, regardless of FEC methodology.

Two main sources of error when counting particles in a liquid (such as nematode eggs in solution) have been identified (Student, 1907). First, the aliquot taken may not be representative of the bulk of the liquid and the distribution of cells over the area that is examined is never uniform, leading to an error of random sampling (Student, 1907; Torgerson et al., 2012). It is has also been proposed that there is no effect of 'interference' between particles, so there is as much chance as of a particle falling in on an area which already has several particles as on one altogether unoccupied (Student, 1907). The aim of the parametric bootstrapping analysis is to encompass such sampling errors in the FECR calculation (Torgerson et al., 2012). The benefit of bootstrapping FECR data in this way is that the sampling distribution is not mathematically generated but is determined from the original FECRT data set (Cabaret and Berrag, 2004). From the data analysed here, for the CF method, with AM *versus* bootstrapping analysis, the sensitivity status concurred for all farms but differed on two occasions when the GM based method was compared to bootstrapping analysis. This would indicate that the AM analysis as conducted here is comparable to the parametric bootstrapping method.

Presently, anthelmintic resistance in UK cattle nematodes is restricted to *Cooperia* spp. surviving ML treatments (Coles et al., 1998; Sargison et al., 2010; McArthur et al., 2011) and, as yet, resistance in other nematode species or resistance to multiple anthelmintic classes has not been identified. However, suspected IVM-resistant *Ostertagi* spp., were identified on three farms in Germany and Sweden following

IVM administration (Demeler et al., 2009), with the caveat that further investigation is required to confirm these findings. Isolates resistant to more than one class of anthelmintic have yet to be reported in Europe, but have been reported elsewhere (Sutherland and Leathwick, 2011). Most notably, in New Zealand, researchers found a predominance of IVM-resistant *Cooperia* spp., with resistance suspected on 56 of 61 beef cattle farms participating in a FECRT (Waghorn et al., 2006) and identified IVM-resistant *Ostertag*ia spp. on four farms and IVM-resistant *Trichstrongylus* spp. on one farm. Overall, resistance to all three classes was identified on four farms, however the species involved on these farms was not reported (Waghorn et al., 2006). It was suggested that farmers use LEV in order to control *Cooperia* spp., as it was the most prevalent genus surviving treatment, but little has been published regarding future control strategies for these drug resistant populations (Waghorn et al., 2006). Further testing on the farms studied here with BZ and LEV products would now be recommended.

As there are currently no new anthelmintic classes available for cattle, further work is required regarding the role of grazing management in the preservation of the efficacy of the three available classes. With concerns raised about efficacy of pour-on preparations (Eagleson and Allerton, 1992), and the prospect of side-resistance developing against other members of an anthelmintic class (Prichard et al., 1980), it is of clinical and epidemiological relevance to know if AR to one anthelmintic product, such as IVM injection tested here, is also observed when using other MLs (such as MOX) and if the results generated after subcutaneous administration differ from those achieved using other application methods. Further evaluation, for

example in the form of a controlled efficacy test, can be used to confirm resistance suspected in the field. The exploration of potential mechanisms behind resistance, with the view to developing better diagnostic technique may help improve the sensitivity of existing techniques. To that effect, two of the isolates generated here were selected for further investigation in a controlled efficacy test and molecular characterisation experiments, reported in Chapters 4 and 5.

Chapter 4: Use of a controlled efficacy test to confirm macrocyclic lactone resistance in two field isolates of *Cooperia oncophora**

*A proportion of the results from this Chapter have been published in a peer-reviewed journal article, see Appendix 3.

4.1 Introduction

The controlled efficacy test (CET) is the current gold standard test for the detection of anthelmintic resistance (Coles et al., 2006). First described in 1941, it requires the necropsy of animals at a set time after anthelmintic administration, having previously been experimentally, or naturally, infected with nematode larvae (Moskey and Harwood, 1941). The reduction in adult worm burdens of treated animals is compared to those of untreated control animals, and so can be used to determine the efficacy of compounds and also the presence of anthelmintic resistance in nematode populations where inefficacy is suspected (Prichard et al., 1980). The CET is considered the definitive and unequivocal test for identifying and confirming anthelmintic resistance (Johansen, 1989) as efficacy can be established against all developmental stages of a wide range of species in a large number of hosts (Wood et al., 1995). This is an advantage over the faecal egg count reduction test (FECRT), which can only provide a measure of anthelmintic efficacy against a patent adult female nematode infection, with measurement of the reduction in FEC following administration (Presidente, 1985). Furthermore, following the CET, adult nematodes can be identified to species level by morphological identification, rather than

generally to genus level as can be achieved when conducting larval differentiation following the FECRT (Prichard et al., 1980).

As reported in Chapter 3, the FECRT can be constrained by relatively low FECs at the time of anthelmintic administration and conducting a FECRT often requires the compliance of farmers to undertake accurate drug administration and faecal sampling, issues that are enhanced by a lack of a standardised protocol for the FECRT in cattle (Sutherland and Leathwick, 2011). Guidelines that outline the procedure of a CET have been published by the World Association for Veterinary Parasitology (WAAVP) (Powers et al., 1982; Wood et al., 1995). The original WAAVP guidelines were published to provide a framework for researchers to use when evaluating the efficacy of new anthelmintic products, to ensure meaningful data was captured and so time, labour and experimental animals could be used efficiently (Powers et al., 1982). The most recent guidelines describing the CET were published in 1995 (Wood et al. 1995) to address advances made since the original guidelines were published in 1982. One such advance was the development of anthelmintics with an efficacy of greater than 98%, such as compounds in the macrocyclic lactone (ML) class. Based on efficacies of earlier classes of anthelmintics, developed in the 1960s and 1970s, the previous guidelines had indicated an efficacy of greater than 90% be classed as “very good” (Wood et al., 1995). The use of a standardised protocol enables derived data to be compared between studies; in particular, data can be compared to CETs that were conducted during the development and registration of an anthelmintic to ascertain efficacy at known dose rates against a known infection of various nematode species (Johansen,

1989) in different regions of the world. The current guidelines recommend that animals selected for use in a CET are of as similar age and breed as possible, and kept under controlled conditions, such as identical diet and housing, to minimise differences associated to external influences between treatment groups (Powers et al., 1982). Each animal is infected with a predetermined number of larvae (within ranges determined adequate to generate a patent infection, but not so high as to cause clinical disease), and is randomly allocated to a treatment group on the basis of factors as weight, age and FEC (Wood et al., 1995). Anthelmintics are applied at the appropriate dose rate (generally the manufacturer's recommended dose rate but different dose rates may be used in original efficacy trials), based on the weight of the individual animal, at a time after parasitic inoculation, which is dependent on the species and developmental stage to be examined. In the case of trichostrongyle nematodes that infect cattle, such as *Ostertagia ostertagi* and *Cooperia oncophora*, a period of 28 to 35 days following infection is deemed optimal for determining efficacy against adult nematodes (Wood et al., 1995). Necropsy is conducted five to seven days following anthelmintic administration, which is of sufficient time to allow the expulsion of paralysed worms but not so long as to allow any unaffected immature stages to develop to adult worms (Powers et al., 1982; Wood et al., 1995). This time period can be used for all classes of anthelmintic where examination of efficacy and not persistence are being examined, unlike the FECRT where the timing of faecal samples collected following anthelmintic administration are dependent on the anthelmintic class used (Coles et al., 1992). Whilst recommendations for statistical analysis are not provided in the guidelines, resistance is proposed as an arithmetic mean reduction in adult worm burden of less than 95%, with a lower 95%

confidence interval of less than 90% (Coles et al., 1992); however, this guideline needs to be clarified depending on which class of anthelmintic is being applied, as some of the earlier classes had a lower observed efficacy when tested in licensing trials (Rubin et al., 1965; Callinan and Cummins, 1979).

Whilst the use of experimental animals and the associated time and labour costs precludes the use of the CET on a frequent basis (Johansen, 1989), it can be utilised to confirm the results of previously conducted FECRTs; a method proposed as the simplest test to perform in the field, but which is not without issue, as described in Chapter 3. As demonstrated with the FECRTs conducted in Chapter 3, and in previously published studies, low FECs at the time of anthelmintic application can lead to difficulties in interpretation of the results (Sutherland and Leathwick, 2011). Furthermore, the usefulness of the FECRT is limited, as it has been indicated that, in the ovine nematodes *Trichostrongylus colubriformis* and *Ostertagia* spp., benzimidazole resistance cannot be reliably detected at levels below 25% (Martin et al., 1989). In addition, anthelmintic administration is known to suppress production and release of eggs from female nematodes for a number of days after administration in a variety of nematode species (Scott et al., 1991; McKenna, 1997). As a result, it can be impossible to determine if a reduction in FEC is a result of the removal of adult nematodes or due a temporary suppression of egg development or oviposition (Vermunt et al., 1996; McKenna, 1997).

The first case of anthelmintic resistance in cattle nematodes to be confirmed by CET was published in 1986 (Eagleson and Bowie, 1986), when an isolate of *Trichostrongylus axei* was confirmed to be resistant to oxfendazole (OXF), following

worm burden analysis of calves 14 days after anthelmintic administration. Compared to the geometric mean worm burden of an untreated control group, a 13% reduction in adult *T. axei* worm burden was observed following oral administration of OXF (Eagleson and Bowie, 1986). In a second group of calves, 100% reduction in adult worm burden was recorded following application with subcutaneously administered ivermectin (IVM, 0.2mg kg^{-1} body weight; BW) (Eagleson and Bowie, 1986). The CET has subsequently been used to confirm cases of IVM and moxidectin (MOX) resistance observed in cattle nematodes in the field and following artificial infections (Vermunt et al., 1995; Gasbarre et al., 2009; Edmonds et al., 2010; de Graef et al., 2012). In the UK, a CET was used to confirm the findings of a FECRT study where IVM resistance was suspected (Coles et al., 1998; Stafford and Coles, 1999). There, ten calves were infected with a suspected IVM resistant *Cooperia* spp. isolate, and five were administered with an IVM injectable formulation (0.2mg kg^{-1} BW) at 28 days following infection. All calves were necropsied seven days after anthelmintic administration and adult *Cooperia* burdens were found to be reduced by 16% in treated animals compared to controls, confirming the presence of resistance in this isolate (Stafford and Coles, 1999). To date, no CETs have been conducted in the UK to examine anthelmintic resistance against moxidectin (MOX) or IVM delivered by other application methods such as pour-on or oral formulations. In Belgium, IVM and MOX resistance were confirmed in a field isolate of *C. oncophora,* with 38% and 31% reductions in adult worm burden recorded against injectable IVM and MOX, respectively (de Graef et al., 2012). In previously conducted FECRTs, FEC analysis conducted 14 days following administration of injectable IVM, showed reductions in *C. oncophora* FEC of 73%,

40% and 0% over successive years, from 2006 to 2008 (El-Abdellati et al., 2010b). Results of the FEC analysis from the CET, using the same IVM product, revealed a 55% reduction in FEC and 38% reduction in adult *C. oncophora* worm burden, confirming the presence of IVM resistance (de Graef et al., 2012).

The anthelmintic application method chosen to ascertain efficacy is of particular importance to studies concerning cattle nematodes as anthelmintic efficacy is considered to be closely linked to pharmacokinetic behaviour (Campbell and Benz, 1984) and changes in anthelmintic formulation may influence uptake, biotransformation and/or presentation (Lanusse et al., 1997; Lifschitz et al., 1999b). Previous studies have shown that alteration of an anthelmintic formulation can affect the pharmacokinetics of the product (Lo et al., 1985). For example, use of an aqueous vehicle for subcutaneously applied IVM was found to allow greater bioavailability than that of a non-aqueous vehicle, with plasma concentrations of 84 ng ml^{-1} IVM compared to 25 ng ml^{-1} IVM two days after administration (Lo et al., 1985). Consequently, this alteration in formulation allowed the aqueous product to be more extensively absorbed and distributed (Lo et al., 1985). With regard to ML products, there have been concerns as to the relative efficacy of ML products when administered as a topical pour-on formulation compared to anthelmintics administered orally or by subcutaneous injection (Leathwick and Miller, 2013). Previously, IVM was demonstrated to be similarly efficacious when administered orally compared to injected subcutaneously, with a mean 100% reduction in adult *O. ostertagi* reported compared to nematode burdens in control animals (Armour et al.,

1980). With adult *C.oncophora*, reductions of 99% and 100% were achieved using the injectable and oral IVM formulations, respectively (Armour et al., 1980).

The premise of a pour-on method of application for anthelmintics was first developed for levamisole (Guerrero et al., 1984). When developed for IVM application, efficacy against both *O. ostertagi* and *C. oncophora* was found to be high (Alva-Valdes et al., 1986), with 100% reductions in adult burdens observed against both nematode species compared to burdens in challenged control aninals. In other studies, variable results were recorded (Bisset et al., 1990; Eagleson and Allerton, 1992). In one trial, considerable differences in efficacy were observed among species; notably, a 100% reduction in adult *O. ostertagi* burdens observed in the same trial in which a 23% reduction was observed in adult *C. oncophora* (Bisset et al., 1990). As there was no history of IVM useage on the farm tested, the authors proposed this was not a case of resistance, but may be a result of *C. oncophora* being a proposed 'dose-limiting species' for IVM, requiring greater concentrations of the anthelmintic to achieve efficacy (Egerton et al., 1979). It was also suggested that as the calves were necropsied 14 days after administration, the adult *C. oncophora* may have recovered from a temporary paralysis caused by IVM within this period, allowing the nematodes to become re-established further down the small intestine (Bisset et al., 1990), a finding which concurs with observations in sheep infected with *Cooperia curticei* (Bogan and McKellar, 1988).

In addition to the confirmation of anthelmintic resistance, material collected following necropsy in a CET can be used for phenotypic and genotypic analyses. The phenotypic characteristics of the surviving worms can be examined; for

example, nematode length and the number of eggs *in utero* in female worms (Kloosterman, 1971; Bairden et al., 1992; de Graef et al., 2012). Work conducted with the ovine nematode, *C. curticei*, showed that female nematodes surviving IVM administration had reduced numbers of eggs present *in utero* compared to worms in untreated animals (McKellar et al., 1988). These results indicate that whilst adult nematodes survive anthelmintic administration, there may be ramifications for egg reproduction (McKellar et al., 1988) .

Surviving worms can also be subjected to genotypic analyses; for example, to identify molecular mechanisms or markers for resistance. MLs have been shown to bind to glutamate-gated chloride (GluCl) channels to exert a paralysing effect upon nematodes (Arena et al., 1991; Arena et al., 1992). The channel is held open by ML binding, allowing an influx of chloride ions which irreversibly hyperpolarise the cell, leading to a rapid paralysis of nematode movement and reduction in pharyngeal pumping (Cleland, 1996; Wolstenholme, 2011) The GluCl receptor is made up of five subunits, arranged around a central pore, as confirmed by X-ray crystallography (Hibbs and Gouaux, 2011). The subunits composing the GluCl channel may be homomeric and comprised of five identical subunits, or heteromeric and comprised of different subunits, some of which may be parasite specific (i.e. not found in the free-living nematode *Caenorhabditis elegans,* (Yates et al., 2003). To date, work in this area has been primarily focused on *C. elegans* and the ovine parasitic nematode *Haemonchus contortus*; however, recent work using isolates of *C. oncophora* that were demonstrated to be IVM resistant *in vivo* has shown differences in the nucleotide sequence of a GluCl subunit (*glc-6*) after exposure to IVM administration

(de Graef et al., 2013a). Genetic analysis was conducted on eight *C.oncophora* isolates for changes in nucleotide sequence in this subunit: three geographically distinct IVM-sensitive populations and two isolates shown to be IVM-resistant (El-Abdellati et al., 2010b), (de Graef et al., 2012) and three populations of IVM-resistant worms exposed to IVM or MOX *in vitro* (de Graef et al., 2013a). Differences in nucleotide sequence between resistant nematodes after anthelmintic exposure were identified near the N terminal extracellular domain of the gene, in the region of the signal peptide (de Graef et al., 2013a).

In this chapter, a CET was performed, first, to confirm the results of the FECRT for two nematode isolates obtained in the studies described in Chapter 3. These isolates were indicated to contain IVM resistant *Cooperia* spp. larvae based on the mean FECR observed following administration of an injectable IVM formulation. Second, the CET was employed to compare the efficacy of IVM and MOX pour-on formulations against the two isolates and to compare the results to those observed with the injectable IVM formulation. Adult *C. oncophora* nematodes recovered at necropsy from untreated control animals and those surviving anthelmintic administration were used for phenotypic and genotypic analyses. The phenotype analysis comprised the measurement of female worm length and the enumeration of eggs *in utero* in female nematodes. Genotype analysis was conducted after extraction of RNA from pools of *C. oncophora* adult worms, followed by PCR amplification and cloning of a region of the glutamate-gated chloride channel gene (*glc-6*) (Glendinning et al., 2011; de Graef et al., 2013a). Sequence comparison was made using material from male and female *C. oncophora* that were recovered from

untreated control calves, and between male and female worms that had survived administration with an IVM injectable formulation. The overall aims were to confirm the presence of IVM resistance in the two field isolates by CET, to investigate the impact application route had on efficacy and to examine mechanisms potentially associated with ML resistance.

4.2 Materials and Methods

4.2.1 Generation of nematode isolates for investigation

In Chapter 3, the results of 21 FECRTs were reported following administration with IVM. The presence of potentially IVM-resistant nematodes was identified on 13 farms, with *Cooperia* spp. being the most prevalent nematode genus identified following treatment on all occasions. Of the 13 farm isolates, two were selected for further research: one derived from Farm 001 and one from Farm 004, namely 004a (McArthur et al., 2011). These isolates are referred to hereafter as field isolates 001 and 004 (FI001 and FI004, respectively). These isolates were selected as both appeared to be IVM resistant by FECRT (Chapter 3) and more than 50, 000 L_3 were generated from the Day 0 coprocultures, producing sufficient larvae to infect a donor calf. A summary of results from the FECRT are displayed in Table 21. To generate sufficient third stage larvae (L_3) to infect 20 calves with each isolate, two donor calves were experimentally infected, one per isolate. To prevent the introduction of any additional anthelmintic selection pressure to these isolates, larvae used to infect the donor calves were derived from samples collected prior to the administration of IVM in the FECRT. A male Holstein Friesian helminth-naïve calf was administered with 50, 000 L_3 *per os* for each isolate and faecal egg count (FEC) analysis was conducted weekly to monitor egg excretion (Jackson, 1974), once infection was observed to be patent, 21 days after infection, calves were harnessed to collect faeces

and collection bags changed twice daily. Larval culture was conducted as described previously in Chapter 3.

Table 21 Provenance of nematode isolates examined in the CET. FECRT calculation, is based on arithmetic mean reduction in FEC 14 days after IVM administration (Coles et al., 1992). Genus composition data is presented based on examination of L_3 obtained prior to the conduction of the FECRT and following passage through a helminth-naïve calf. On each occasion, 100 randomly selected L_3 were morphologically identified to genus level.

Isolate	Location	Percentage reduction in faecal egg count (95% confidence interval)	Percentage genus composition of larvae prior to the on farm FECRT	Percentage genus composition following experimental passage through a helminth-naïve calf
FI001	Dumfriesshire, Scotland	72 (15 – 91)	85% *Cooperia* spp. 15% *Ostertagia* spp.	80% *Cooperia* spp. 20% *Ostertagia* spp.
FI004	East Ayrshire, Scotland	87 (69 – 95)	62% *Cooperia* spp. 38% *Ostertagia* spp.	60% *Cooperia* spp. 40% *Ostertagia* spp.

A test of equal proportions (Newcombe, 1998) was conducted on the larval genus results following nematode passage through the helminth-naïve calves to assess changes in genus proportions, which was not found to be statistically significant for either isolate ($P > 0.45$).

4.2.2 Experimental design of controlled efficacy test (CET)

Forty, helminth-free male dairy calves, housed under conditions to minimise parasitic nematode infection, were administered *per os* with 50,000 L_3 from isolate

FI001 (80% *Cooperia* spp., 20% *Ostertagia* spp.) or FI004 (60% *Cooperia* spp., 40% *Ostertagia* spp.). Calves ranged from four to seven months in age and weighed between 90 and 185 kg. Prior to experimental infection, faecal samples were taken on two occasions to confirm that no patent helminth infections were present. On day 27 post-infection (PI), all calves were weighed, faecal sampled and allocated to one of four treatment groups per isolate. Group allocation was based on weight, age and FEC, with five calves per treatment group, as described in Table 22. A power calculation was used to determine group size and group sizes were deemed be to be appropriate by ethical review (Moredun Research Institute Experiments Committee). Calves were administered anthelmintic treatment at the manufacturers' recommended dose rates on day 28 PI in the following treatment groups: IVM injectable (Ivomec Super®, 1% w/v IVM, 10% w/v clorsulon, 0.2 mg kg^{-1} body weight; BW; Merial Animal Health), IVM pour-on (Ivomec Pour-On®, 0.5% w/v IVM, 5 mg kg^{-1} BW; Merial Animal Health) and MOX pour-on (Cydectin Pour-On ®, 0.5% w/v MOX, 5 mg kg^{-1} BW; Pfizer Animal Health Ltd). Dose rates for injectable IVM administration were rounded up to the nearest 0.1 ml and applied subcutaneously with 17 ½ gauge needle as per manufacturer's instructions. All pour-on administrations were rounded up to the nearest 1 ml and applied using a 20 ml syringe along the midline of the back, from withers to tail head. For each isolate, one group was used as an anthelmintic untreated control group. The eight groups were housed separately and the calves that had received a pour-on application were observed for 30 min following administration for any evidence of licking behaviour. All experimental procedures were approved by the Moredun Research Institute Experiments and Ethics Committee and conducted under the legislation of a UK

Home Office License (reference PPL 60/03899) in accordance with the Animals (Scientific Procedures) Act of 1986.

Table 22: Anthelmintic treatment groups for calves on trial in the CET. Anthelmintic dosage rates are displayed as mg per kg bodyweight (BW). Injectable and pour-on applications are represented by "INJ" and "PO" respectively. Ivermectin and moxidectin are represented by "IVM" and "MOX" respectively.

Anthelmintic application (dosage)	Designation	
	Field Isolate 001	Field Isolate 004
Injectable ivermectin(0.2 mg kg^{-1} bodyweight; BW)	IVM-INJ-01	IVM-INJ-04
Pour-on ivermectin (0.5 mg kg^{-1} BW)	IVM-PO-01	IVM-PO-04
Pour-on moxidectin (0.5 mg kg^{-1} BW)	MOX-PO-01	MOX-PO-04
None (i.e. untreated control group)	CONTROL-01	CONTROL-04

4.2.3. Collection of parasitological material during controlled efficacy test

4.2.3.1 Faecal egg count (FEC) samples

Rectal faecal samples were collected throughout the CET experiment for FEC analysis; one day prior to treatment (day 27 PI) for purposes of treatment group allocation; and then daily until the end of the experiment (day 35 PI). All samples were taken at the same time of day and two FEC methodologies were conducted in duplicate for each sample: a centrifugal flotation method (Jackson, 1974) and a

McMaster method (Gordon and Whitlock, 1939) as described in Chapter 3, Section 3.2.2.1.

4.2.3.2 Collection and processing of blood plasma samples

Blood samples were collected from all calves prior to anthelmintic administration via jugular venepuncture into 10 ml heparinised Vacutainer® blood collection tubes (Becton, Dickinson and Company). Blood samples were then taken from all treated calves at 4, 8, 24, 48, 120, 144 and 168 h after anthelmintic administration. Immediately after collection, the sample tubes were inverted several times and placed inside a lidded cool box to prevent ML degradation due to sunlight (Halley et al., 1989). Blood samples were centrifuged within 1 h of collection at 1275 x *g* for 15 min at 4 °C. The separated plasma was then aliquoted into dark amber Eppendorf tubes and stored at -20 °C until analysis.

4.2.3.3 Analysis of ML concentration in blood plasma

Determination of ML concentrations in the plasma samples was conducted by Mr Jean-François Sutra and Dr Anne Lespine (INRA, Toulouse, France). IVM and MOX concentrations were determined in plasma by high performance liquid chromatography (HPLC) with fluorescence detection according to previously described and validated methods (Alvinerie et al., 1995; Alvinerie et al., 1998). Data were analysed using a non-compartmental approach with version 4.2 of the Kinetica Tm computer program (InnaPhase, Philadelphia, USA). Data are expressed as arithmetic mean and standard error of the arithmetic mean (S.E.M.). The partial area

under the plasma concentration–time curve (AUC) was calculated by the linear trapezoidal rule (Yeh and Kwan, 1978).

4.2.3.4 Organ processing following calf necropsy

At necropsy (35 days PI, 7 days following anthelmintic administration), the abomasum and entire length of the small intestine were removed from each calf. The abomasum was opened along the greater curvature and deposited, together with the abomasal contents, into a labelled 10 L bucket. The mucosa was gently washed with lukewarm physiological saline (0.85% NaCl; 85 g NaCl, 10 L tapwater) and added to the bucket of abomasal contents. Additional physiological saline was added to the bucket to a volume of 10 L and incubated for 4 h at 37 °C (Powers et al., 1982; Patterson et al., 1996). The small intestine was treated similarly, with the intestinal contents collected into a bucket, the full length of the intestine opened and added to the bucket; warm physiological saline added up to a volume of 10 L and incubated as per the abomasum. After 4 h, the organs were removed from their respective buckets and the surface mucosa rubbed to remove any adhering worms, with particular attention paid to cut surfaces and tissue folds. The contents and digest were replenished to 10 L with warm physiological saline. Following thorough agitation, a 10% subsample (1 L) was removed and fixed using 100 ml 100% formalin (40% v/v formaldehyde; Fisher Scientific). From this 10% subsample, a 220 ml subsample, equivalent to 2% total volume, was removed and stained with 10 ml helminthological iodine (250 g potassium iodine, 50 g iodine, 500 ml distilled water; Sigma Aldrich). The remaining 9 L organ contents were left to sediment for 15 min, after which the supernatant was removed and the sedimented contents fixed with 100% molecular

grade ethanol (EtOH; Fisher Scientific) to a final concentration of greater than 70% EtOH. Formalin-fixed subsamples were washed over a 38 µm sieve to remove excess iodine before all nematodes were removed and enumerated. Nematodes were classified according to sex, stage of development and species using the Ministry of Agriculture, Fisheries and Food document guidelines (M.A.F.F., 1986). EtOH fixed subsamples were treated similarly, with nematodes classified in accordance to sex and species and transferred to bijoux containing 100% EtOH.

4.2.3.5 Examination of female *Cooperia oncophora* length and numbers of eggs *in utero*

For each animal, up to 25 randomly selected formalin-fixed adult female *C. oncophora* were mounted on glass slides with lactophenol (Sigma Aldrich), photographed immediately under x 40 magnification (Nikon D90 camera), followed by enumeration of eggs *in utero* under x 100 magnification on a stereomicroscope. Digital images were downloaded into Image-Pro Express software (version 5.0.1.26, Media Cybernetics, Inc.). This software was used to measure the length of each female nematode and calibrated after every 25 nematodes by measurement of a 1 mm graticule under x 40 magnification.

4.2.3.6 Statistical analysis

Small intestinal nematode burdens were checked for normality and differences between treatment groups compared using two-sample t-tests. A one-way ANOVA test was used to assess if the daily fluctuations in FEC were statistically significant..

Percentage efficacy was calculated using mean values of (1-(T/C) x100) (where C is mean nematode burden of the control group and T is mean nematode burden of the anthelmintic treated group (Coles et al., 1992). Data manipulation and graphs were drawn in Microsoft Excel 2007 and statistical analyses performed using R Statistical Environment (R Core Team, 2013). Arithmetic mean estimates were calculated by adding the FEC values together and dividing by the number of samples present. Geometric means were also calculated by the anti-log of log-transformed counts (Dash et al., 1988). In order to calculate geometric means, a nominal value of one was added to each EPG prior to analysis and subsequently removed following analysis, as is accepted practice (Fulford, 1994). Unless explicitly stated otherwise, all mean values are presented as arithmetic means.

4.2.3 Molecular analysis

4.2.3.1 Ribonucleic acid (RNA) extraction protocol

Ribonucleic acid (RNA) was extracted from eight aliquots containing 10 adult *C. oncophora* nematodes, fixed in EtOH at post-mortem, as shown in Table 23. The RNA samples were used for cloning and sequence analysis. Two male *C. oncophora* were randomly selected from EtOH-fixed subsamples from each of the five calves in CONTROL-01, resulting in a pool of 10 worms in total.

Table 23: Provenance of nematodes used for RNA extraction and molecular sequence analysis. All nematodes were recovered 35 days post infection (PI). Nematodes selected from untreated control groups are denoted "CON", with nematodes surviving IVM administration, seven days after application are denoted "IVM". Male and female nematodes are denoted "M" and "F" respectively, and field isolates 001 and 004 are denoted by "01" and "04", respectively.

Treatment group	Field Isolate 001	Field Isolate 004
Untreated, control calves	10 female *C. oncophora* (CON-F-01)	10 female *C. oncophora* (CON-F-04)
	10 male *C. oncophora* (CON-M-01)	10 male *C. oncophora* (CON-M-04)
IVM injectable (day 7 following IVM administration)	10 female *C. oncophora* (IVM-F-01)	10 female *C. oncophora* (IVM-F-04)
	10 male *C. oncophora* (IVM-M-01)	10 male *C. oncophora* (IVM-M-04)

A pool of 10 female *C. oncophora* was selected in the same manner for CONTROL-01, and the entire process repeated for CONTROL-04, and for groups IVM-INJ-01 and IVM-INJ-04. The adult nematodes were transferred from EtOH storage using a sterile pipette tip to a 2 ml ribolyser tube containing ceramic beads (Precellys$^{(R)}$ soft-tissue homogenising CK-14 tubes, Bertin Technologies), and chilled on ice. To each tube, 1 ml TRIzol reagent (Invitrogen) was added and the nematodes homogenised using a Precellys$^{(R)}$ 24 machine (Bertin Technologies), utilising a programme consisting of two homogenisation cycles of 30 s at 6500 rpm, with 2 min on ice between cycles to prevent RNA degradation. After this, the tubes were examined to ensure homogenisation was complete. Tubes were incubated at room temperature for 5 min and the TRIzol (Invitrogen) protocol followed as per manufacturer's instructions. Following extraction, RNA was subjected to DNAse treatment (Promega) and the concentration of the RNA estimated by spectrophotometer

(Nanodrop, ThermoScientific). The remaining RNA (~19 µl) was divided into four 5 µl aliquots and stored at -80 °C.

4.2.3.2 Amplification of partial glutamate-gated chloride channel gene (*glc-6)*

Initial analysis focused on generating full-length complimentary DNA (cDNA) sequence for the GluCl gene, *glc-6*, using RNA from 10 *C. oncophora*; however, inconsistent amplification (data not shown) led to the design of a more targeted approach, which focussed on a region of the *glc-6* cDNA, previously identified to show differences in sequence between IVM-resistant *C. oncophora* prior to anthelmintic administration and those surviving seven days after anthelmintic administration to calves (de Graef et al., 2013a). Glyceraldehyde 3-phosphate dehydrogenase (*gapdh*) was selected as the positive control template (Van Zeveren et al., 2007b; El-Abdellati et al., 2011; de Graef et al., 2013b). The primer sequences used in the PCR experiments are listed in Table 24. All primers were generated by MWG Eurofins and internal primer selection verified by Primer3 software (Untergrasser et al., 2012).

Table 24: List of primer sequences used in the molecular analyses

Gene name	Amplification purpose	Primer name	Primer Sequence (5' -> 3')
Gapdh	Positive control	GapdhF GapdhR	TCAAGGTCCACAACAGCAAG CGTTGTAGGTCTCATTTGTTT
Glc-6	One-step reverse transcriptase PCR	$Glc6F_{Full}$ $Glc6R_{Full}$ $Glc6F_{internal}$ $Glc6R_{internal}$	GCTTATCATAGTTTTCGGGAGT TACCACGTCCAGTAGAAGAT CATCTTGACAAGTGACGTTGAT AATGTATCCGGCGTCCAGAT
Vector primers	Cloning	SP6 T7	ATTTAGGTGACACTATAGAA GTAATACGACTCACTATAGGGC

4.2.3.2.1 Optimised PCR protocol for partial *glc-6* amplification

A one step reverse transcriptase (RT)-PCR procedure was followed using the SuperScript™One-Step RT-PCR kit (Invitrogen) in combination with Platinum® Taq (Invitrogen) following manufacturer's instructions. PCR reaction mixes had final concentrations of 0.5 µl RNA template, 0.8 µM of both forward and reverse primers, 1 unit of RT/Platinum® Taq Mix, in a reaction buffer containing 0.2 mM of each dNTP and 1.2 mM $MgCl_2$, in a total volume of 25 µl. For the purposes of cDNA synthesis, the reactions were incubated for 30 min at 50°C followed by 2 min at 94°C. The PCR reaction followed directly, with 40 cycles of denaturing (30 s at 94 °C), annealing (30 s at 50 °C) and elongation (90 s at 72 °C), with a final elongation incubation for 10 min at 72 °C. In all cases, positive and negative control reactions were included; with *gapdh* primers used for the positive control and 'no template' reactions for the negative controls. Negative controls consisted of both *glc-6* and *gapdh* reactions with 1 µl nuclease-free water in place of template. Following the PCR step, amplification products were visualized by gel electrophoresis on 1.5% agarose gels with GelRed™ nucleic acid stain (Cambridge

Bioscience). To 1 µl blue-orange loading dye (Promega), 4 µl PCR products were added, loaded into a well and a voltage of 80 volts applied for 40 min. A 100 base pair (bp) ladder (Promega) was included each time to estimate amplicon size.

4.2.3.3 Cloning of partial glutamate-gated chloride channel gene (glc-6)

PCR amplification products of an appropriate size (319 bp) were purified using the Qiaquick PCR purification kit (Qiagen), followed by overnight ligation in pGEM-T Easy vector (Promega) and transformed into JM109 *Escherichia coli* competent cells (Promega) according to the manufacturer's instructions. Transformed plasmids were incubated for 90 min at 37 °C in a shaking incubator in the presence of SOC medium (20 g L^{-1} Tryptone, 5 g L^{-1} Yeast Extract, 4.8 g L^{-1} $MgSO_4$, 3.6 g L^{-1} dextrose, 500 mg L^{-1} NaCl, 186 mg L^{-1} KCl; Bioline UK) after which, 20µl or 200 µl of each transformation were spread on LB medium (15 g L^{-1} agar, 10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 5 g L^{-1} NaCl) plates containing ampicillin (100 µg ml^{-1}; Sigma Aldrich), IPTG (0.5 mM; Isopropyl β-D-1-thiogalactopyranoside; Promega) and X-Gal (80 µg ml^{-1}; 5-bromo-4-chloro-3-indolyl-β-D-galactoside; Promega). Plates were incubated overnight at 37 °C, and colonies for sequencing were selected on a blue - white basis. For each plate, five white colonies were picked, resulting in a total of 10 colonies per transformation. Colony PCR was performed using T7 and SP6 primers, in a 50 µl reaction, with one unit Platinum Taq (Invitrogen), 1 mM $MgCl_2$, 0.2 µM of each primer and 0.2 µM of each dNTP per reaction. The PCR conditions were an initial step of 94 °C for 5 min, followed by 30 cycles of denaturing (30 sec at 94 °C), annealing (30 sec at 55 °C) and elongation (60 s at 72 °C) with a final elongation incubation for 7 min at 72 °C. The presence of an insert of the correct size was confirmed by gel electrophoresis as above. Colonies that

contained an insert of the correct size were amplified in 10 ml LB+ampicillin (150 µg ml^{-1}) media, for 14 h at 37 °C in an orbital shaking incubator. Plasmids were purified using the Wizard plus SV kit (Promega) and sequenced on both forward and reverse strands (Eurofins MWG) using SP6 and T7 primers (Table 4.2), resulting in the generation of 160 sequences. The sequence data was analysed using Lasergene 10 software (DNAstar) and searched against BLASTn database (NCBI – http://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm identity. Phylogenetic and multiple pairwise alignments were conducted in Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo) and a neighbour-joining cladogram constructed in Clustal W2 (https://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny).

4.3 Results

4.3.1 Controlled efficacy test

4.3.2 Analysis of blood plasma to determine macrocyclic lactone concentration

For the duration of the observation period, none of the calves administered with a pour-on application were observed to lick either themselves or other calves within the same treatment group.

There was no IVM detected in plasma samples obtained from one of the calves (animal number 302354) infected with FI001 and administered with IVM injectable formulation (group IVM-INJ-01). As a result, all data from this animal were excluded from analysis. The plasma profiles of the anthelmintic formulations are shown in Figure 20. The observed peak concentration (C_{max}) was seen to differ between treatment groups and between isolates.

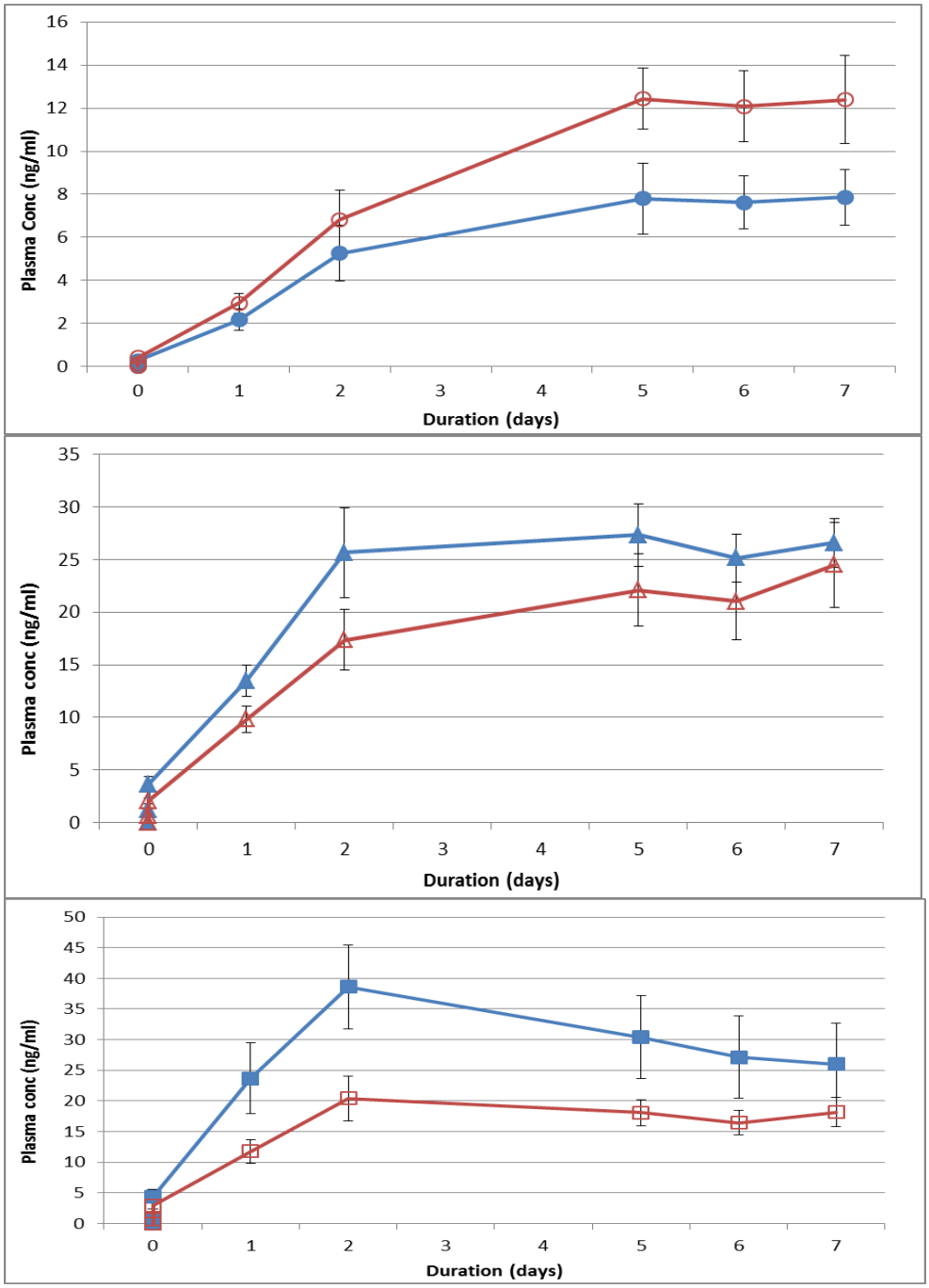

Figure 20: Concentration of ML plasma concentration (ng ml^{-1}) for each treatment group over the duration of the trial, error bars represent the standard error of the mean. Filled and open symbols represent calves infected with FI001 and FI004, respectively. The top pane comprises groups MOX-PO-01 and MOX-PO-04, the middle pane contains groups IVM-PO-01 and IVM-PO-04, and the bottom pane, IVM-INJ-01 and IVM-INJ-04. Groups administered injectable IVM are identified by square symbols (□); IVM pour-on recipients by triangle symbols (Δ) and MOX pour-on recipients by circles (○).

The IVM concentrations present in plasma taken from calves in group IVM-INJ-01 were seen to increase for up to two days following administration, when a mean peak of 39 ng ml^{-1} IVM was observed (± 7 standard error of mean, SEM; range 18 – 50 ng ml^{-1} IVM). By necropsy, seven days after administration, IVM concentrations had decreased to a mean of 26 ng ml^{-1} IVM (± 7 SEM; range 10 – 42 ng ml^{-1}). IVM concentrations in plasma from calves in group IVM-PO-01 peaked at a mean concentration of 27 ng ml^{-1} IVM (± 7 SEM; range 19 – 35 ng ml^{-1}) five days after administration. The mean IVM concentration was found to have decreased slightly at six days after administration (25 ng ml^{-1} IVM, ± 3 SEM; range 19 – 35 ng ml^{-1}), before increasing to a mean concentration of 27 ng ml^{-1} IVM (± 2 SEM; range 19 – 34 ng ml^{-1}) at necropsy. Compared to the IVM plasma results, lower plasma concentrations were observed from the calves infected with FI001 and administered with MOX pour-on product (group MOX-PO-01). The mean peak MOX values were observed five days after administration (8 ng ml^{-1} MOX ± 1 SEM; range 4 – 11), with similar mean values obtained for the following two days (8 ng ml^{-1} MOX, ± 2 SEM; range 3 – 12).

Plasma taken from calves in group IVM-INJ-04 showed a lower mean C_{max} concentration than in plasma from calves applied with the same product in group IVM-INJ-01. IVM concentrations increased until two days following administration, when a C_{max} of 20 ng ml^{-1} IVM was observed (± 4 SEM; range 9 – 31 ng ml^{-1} IVM). By necropsy at seven days after administration, C_{max} had decreased to a mean of 18 ng ml^{-1} IVM (± 2 SEM; range 11 – 24). The C_{max} in plasma from calves in group

IVM-PO-04 was observed seven days after administration, compared to five days as observed with group IVM-PO-01. A mean peak IVM concentration of 25 ng ml^{-1} IVM (± 4 SEM; range 10 – 35) was observed. Similar values were observed over the preceding two days, with mean IVM concentrations of 22 ng ml^{-1} IVM (± 3 SEM; range 12 – 33) and 21 ng ml^{-1} IVM (± 4 SEM; range 9 – 30) observed five and six days after administration, respectively. Finally, for plasma from calves in group MOX-PO-04, the mean C_{max} value was observed five days after application (12 ng ml^{-1} MOX ± 1 SEM; range 9 – 16), an estimate which was maintained over the following two days, a pattern similar to that observed in plasma collected from calves in group MOX-PO-01.

4.3.1.2. Faecal egg count analysis

Faecal egg count results obtained using a centrifugal flotation method (Jackson, 1974) are presented in Figure 21 and Table 25. All FEC values presented here are the result of an arithmetic mean calculated from two FEC counts from each calf. For calves infected with FI001, the mean FEC on day of treatment (day 0; day 28 post infection) across all four groups, was 1,008 EPG (± 173 SEM; range 252 – 2,624). The mean FEC from the untreated control group on this day was 1,425 EPG (± 471 SEM; range 446 – 2,624). The lowest mean FEC for the control group was recorded on day five following treatment, with 1,036 EPG (± 251 SEM; range 551 – 1,764). FECs then increased until necropsy when the mean FEC was 1,491 EPG (± 422 SEM; range 675 – 2,741). The daily fluctuations in FEC observed in this group for the duration of the CET were not found to be statistically significant, as measured by ANOVA analysis ($P = 0.98$).

On day 0, the mean FEC from calves in group IVM-INJ-01 was 597 EPG (± 91 SEM; range 486 - 869). This rose to a mean of 740 EPG (± 96 SEM; range 455 - 851) one day after application. The lowest mean FEC for this group was recorded five days after IVM administration (14 EPG ± 2 SEM; range 11 – 17), which increased to a mean of 30 EPG (± 9 SEM; range 4 - 44) at necropsy. The mean FEC for calves in group IVM-PO-01 was 1,133 EPG (± 357 SEM; range 491 – 2,453). The mean FEC for this group decreased in the days following anthelmintic application, to a mean of 4 EPG (± 4 SEM; range 0 – 21) after five days, which increased to a mean of 13 EPG (± 11 SEM; range 2 - 57) at necropsy. Calves in group MOX-PO-01 had a mean FEC of 795 (± 268, range 116 – 972) on day of application. The mean FEC decreased following administration to 3 EPG (± 3 SEM; range 0 – 15) after six days, increasing to 4 EPG (± 3 SEM; range 0 - 12) after seven days.

In general, the FECs observed in samples from the calves infected with FI004 were lower than those observed with calves infected with FI001. For all calves infected with FI004, a mean FEC of 613 EPG (± 165 SEM; range 116 – 1,562) was observed on the day of treatment. The untreated control calves had a mean FEC of 613 EPG (± 422 SEM; range 675 – 2,741) on day of treatment. The highest FEC was observed on the third day following anthelmintic application, with a mean of 1,004 EPG (± 182 SEM; range 401 – 1,440) and subsequently decreased to a mean FEC of 515 EPG (± 249 SEM; range 126 – 1,458) by necropsy. As observed with the untreated control calves from FI001, the fluctuations in FEC observed across the

duration of the CET for the FI004 untreated control calves were not found to be statistically significant (P = 0.7).

Calves in group IVM-INJ-04 showed mean FEC values of 782 EPG (± 243 SEM; range 365 – 1,562) on day of administration. The lowest mean FEC was observed four days after the application of anthelmintic (130 EPG ± 40 SEM; range 39 - 279). Seven days after anthelmintic application, the mean FEC had increased to 324 EPG (± 41 SEM; range 221 – 428) at necropsy. FEC values estimates from calves in group IVM-PO-04 increased from a mean FEC of 579 EPG (± 173 SEM; range 116 - 972) on day of treatment, to mean FEC value of 766 EPG (± 147 SEM; range 419 – 1,170) one day after anthelmintic administration. The mean FEC in this group was lowest at five days after treatment (231 EPG ± 51 SEM; range 98 - 392) before increasing to 412 EPG (± 208 SEM; range 113 – 1,215) two days later, at necropsy. Calves in group MOX-PO-04 displayed a mean FEC of 799 EPG (± 178 SEM; range 347 – 1,305) on day of administration. The FECs of this group began to decrease, with a mean of 1 EPG (± 1 SEM; range 0 - 3) by necropsy.

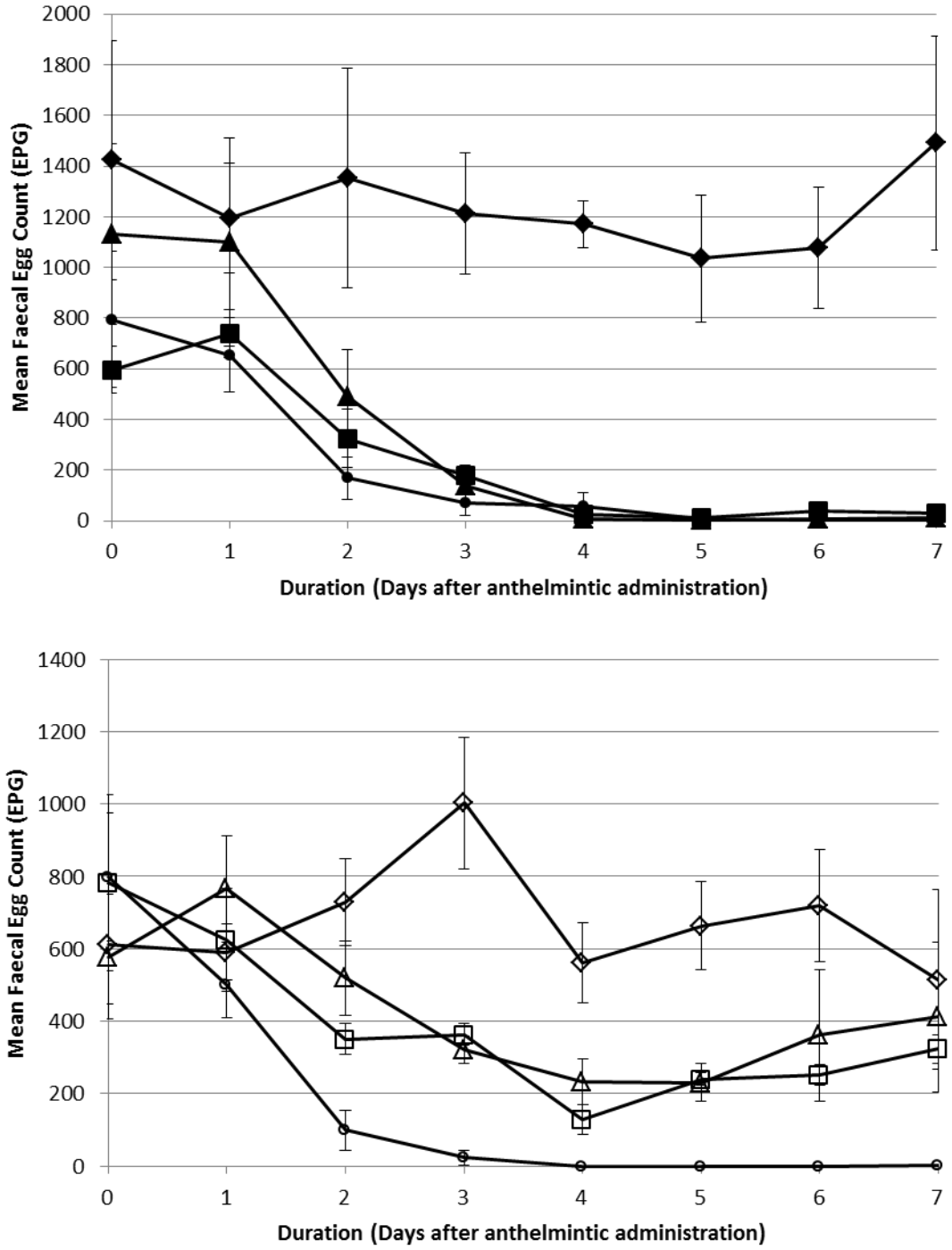

Figure 21: Mean faecal egg count values for isolates FI001 (top pane) and FI004 (bottom pane) during the controlled efficacy test. Error bars indicate the standard error of the mean (SEM). Untreated control groups are indicated with diamond symbols (◊); IVM injectable groups by square symbols (□); IVM pour-on groups by triangle symbols (Δ) and MOX pour-on groups by circles (○).

Two calculations were made to ascertain the percentage reduction in FEC, the results displayed in Table 25. In the first calculation, the mean day 7 FEC for a treatment group was divided by that of the mean day 7 FEC of the respective control group, as described by Coles et al., (1992). In the second calculation, the mean day 0 FEC of each group was divided by the mean day 7 FEC of the same group (Kochapakdee et al., 1995). For calves infected with FI001, reductions in FEC of 98, 99 and 100% were observed for groups IVM-INJ-01, IVM-PO-01 and MOX-PO-01, respectively, regardless of the calculation used. For calves infected with FI004, 100% reduction in FEC was observed with the group MOX-PO-04. With IMV-INJ-04 and IVM-PO-04 groups, the percentage reduction in FEC differed dependent on the calculation used. By comparing the FECs on day 0 and seven days later, percentage reductions of 59% (IVM-INJ-04) and 29% (IVM-PO-04) were observed. These values are higher than those obtained when FECs from the treatment groups were compared to the untreated control group (CONTROL-04), with reductions of 37% (IVM-INJ-04) and 20% (IVM-PO-04) observed using this calculation.

Table 25: Faecal egg count values and percentage reduction estimates for isolates FI001 and FI004 treatment groups. Mean FEC values for day of treatment samples and those taken seven days later are presented with the SEM with the range in brackets. FECR values were calculated either by comparing the arithmetic mean Day 7 FEC of treated groups (T2) to the respective control group (C2; Treated versus Control) or calculated based on the observed reduction in FEC seven days after anthelmintic treatment compared to the arithmetic mean day of treatment FEC (Day 0 (T1) versus Day 7). Estimates represent percentage reduction in egg number and 95% confidence intervals are presented in square brackets

	Mean Faecal Egg Count (EPG **± SEM (range)**)		Percentage reduction in FEC (%) [95% confidence interval]	
Treatment Group	Day 0	Day 7	T2 *versus* C2	T1 *versus* T2
CONTROL-01	1,425 ± 471 (446 – 2,624)	1,491 ± 421 (675 – 2,741)	-	-
IVM-INJ-01	597 ± 91 (456 – 869)	30 ± 9 (4 – 44)	98 [95 – 99]	98 [96 – 99]
IVM-PO-01	1,133 ± 357 (491 – 2,453)	13 ± 11 (2 – 57)	99 [95 – 100]	99 [95 – 100]
MOX-PO-01	795 ± 268 (252 – 1,715)	4 ± 3 (0 – 12)	100 [99 – 100]	100 [99 – 100]
CONTROL-04	613 ± 165 (189 – 1,184)	515 ± 249 (126 – 1,458)	-	-
IVM-INJ-04	782 ± 243 (365 – 1,562)	324 ± 41 (221 – 428)	37 [0 – 77]	59 [0 – 69]
IVM-PO-04	579 ± 172 (116 – 972)	412 ± 208 (113 – 1,215)	20 [0 – 81]	29 [0 – 76]
MOX-PO-04	799 ± 178 (347 – 1,305)	1 ± 1 (0 – 3)	100 [99 – 100]	100 [100 – 100]

There was no change in percentage reduction values when geometric mean FEC values were used in place of the arithmetic mean values presented here. In light of this finding, the results of the geometric mean analysis are not presented.

4.3.1.3. Adult worm burden analysis

4.3.1.3.1 Worm burden analysis of calves infected with FI001

An arithmetic mean total of 13,630 adult nematodes (ranging from 7,950 to 19,400 nematodes) were observed in the abomasum and small intestine of the untreated control calves in group CONTROL-01, equivalent to a percentage establishment of 27%, based on the initial inoculum of 50,000 L_3. All nematodes from the abomasal and small intestinal samples were identified as *O. ostertagi* and *C. oncophora,* respectively, Table 26. The mean number of adult *O. ostertagi* recovered from the calves in CONTROL-01 was 8,280 (± 2,693 SEM; range 4,150 – 18,100). No juvenile *O. ostertagi* worms were observed in any of the abomasal subsamples taken from calves infected with this isolate. In the small intestine, a mean of 5,350 (± 1,288 SEM; range 1,300 – 8,350) *C. oncophora* were detected. Juvenile *C. oncophora* were found in small intestinal subsamples from three calves, with estimates of 50, 100 and 150 (calves 501007, 300948 and 102316, respectively).

Subsamples from two calves in IVM-INJ-01 were observed to have adult *O. ostertagi* present in the abomasum (namely 501014 and 602321), each with 50 adult worms. As a result, for this treatment group, there was 99.7% reduction in adult *O. ostertagi* worm burden compared to the calves in CONTROL-01. Based on the small intestinal subsamples from all calves in IVM-INJ-01, a mean of 3,325 (± 1,175 SEM; range 600 – 6,050) adult *C. oncophora* were found. This equated to a 38% reduction in *C. oncophora* adult nematode burden in comparison to the mean *C. oncophora* burden of calves in CONTROL-01.

There was 100% reduction in adult *O. ostertagi* from calves in group IVM-PO-01, with no nematodes observed in any of the abomasal samples. For the small intestinal

worm burden, a mean of 1,930 (± 1,098 SEM; range 50 – 6,050) adult *C. oncophora* were enumerated. As a result, a 64% reduction in adult *C. oncophora* worm burden was recorded, in comparison to the mean *C. oncophora* burden of CONTROL-01.

For calves in group MOX-PO-01, a 100% reduction in *O. ostertagi* was observed, with no nematodes observed in any of the examined samples. From the five calves in this treatment group, one calf (301106) was observed to have 100 juvenile *C. oncophora* present in the small intestinal subsample. A mean of 3,700 (± 1,818 SEM; range 0 – 8,550) adult *C. oncophora* were calculated in the small intestines of these calves after MOX pour-on administration. Samples from one calf (401027) had no adult or juvenile *C. oncophora* present in the small intestinal subsample. As a group, the calves in group MOX-PO-01 displayed 31% reduction in mean *C. oncophora* adult worm burden compared to that of CONTROL-01 group.

Overall, for calves infected with FI001, the mean number of *C. oncophora* nematodes recovered from each treatment group were not found to be significantly different from the mean number of *C. oncophora* recovered from the untreated control calves ($P > 0.05$). Similarly, there was no significant difference found when the mean *C. oncophora* burden of any group receiving an anthelmintic administration was compared to the other groups in receipt of an anthelmintic administration ($P > 0.05$).

4.3.1.3.2 Worm burden analysis of calves infected with FI004

Based on the mean nematode burden of calves in group CONTROL-04, a mean establishment rate of 35% was observed, based on the initial inoculum of 50,000 L_3,

with a mean of 17,560 adult nematodes being recovered, ranging from 8,200 to 28,650 nematodes per calf. For all calves infected with this isolate, no juvenile *O. ostertagi* were observed in abomasal samples, nor were any juvenile *C. oncophora* observed in small intestinal samples.

The calves in group CONTROL-04 had a mean *O. ostertagi* burden of 15,120 (± 3,549 SEM; range 7, 200 – 25,900). The mean *C. oncophora* adult burden was 2,440 (± 649 SEM; range 850 – 3,900). Using the two-sampled t-test, these figures were not found to be significantly different from those derived from the calves in CONTROL-01 ($p > 0.05$).

In group IVM-INJ-04, one calf (701009) was found to contain *O. ostertagi* in the abomasal sample, numbering 200 nematodes. For this group, there was a mean 99.7% reduction in adult *O. ostertagi* compared to the mean *O. ostertagi* burden from calves in CONTROL-04. In the small intestine, a mean of 1,160 (± 73 SEM; range 1,000 – 1,350) *C. oncophora* were calculated. Compared to the mean *C. oncophora* burden of the calves in CONTROL-04, this equated to 10% reduction in worm burden following injectable IVM administration.

In four of the five calves in group IVM-PO-04, no *O. ostertagi* were present in the abomasum, with one calf (701006) containing 50 adult *O. ostertagi*. As a result, the mean percentage reduction of *O.ostertagi* in this treatment group was 99.9%, compared to the mean *O.ostertagi* burden of CONTROL-04. For the small intestinal worm burdens, a mean of 1, 430 (± 167 SEM; range 1, 100 – 1, 950) adult *C. oncophora* were calculated. This equated to a zero percent reduction in mean

number of *C. oncophora* present, compared to the mean number of *C. oncophora* in the calves from CONTROL-04.

No *O.ostertagi* were observed in abomasal samples from calves in group MOX-PO-04, therefore 100% reduction in *O. ostertagi* was recorded. The mean number of *C. oncophora* recorded was 290 (± 97 SEM; range 100 – 650). When compared to the numbers of *C. oncophora* calculated in the untreated control calves, this equated to a 74% reduction in *C. oncophora* worm burden. This was the greatest reduction in *C. oncophora* worm burden observed in any of the six groups receiving an anthelmintic application.

Overall, for calves infected with FI004, there were no significant differences in mean *C. oncophora* worm burdens between the untreated, control calves (CONTROL-04) and any of the groups administered anthelmintic ($P > 0.05$). However, a significant difference was observed between the mean *C.oncophora* burden of the group MOX-PO-04 and group IVM-INJ04 and IVM-PO-04 (P values < 0.008). There was no significant difference between mean *C. oncophora* burdens of groups IVM-INJ-04 and IVM-PO-04 ($P > 0.05$).

Table 26: Small intestinal worm burden results for each isolate, shown as arithmetic mean, including S.E.M for each group and the range in nematode burden, in addition to the percentage efficacy of each group compared to the respective control group The percentage of male to female *C. oncophora* (M:F) is also presented.

	Mean small intestinal worm burden ± SEM [range]					
Treatment group	Male	Female	Juvenile	Total	Percentage Efficacy	Percentage M:F
CONTROL-01	2,480 ± 635 [400-3,800]	2,810 ± 633 [900-4,400]	60 ± 29 [0-150]	5,350 ± 1,288 [1,300-8,350]	NA	46:53
IVM-INJ-01	1,425 ± 429 [400-2,600]	1,900 ± 625 [200-3,450]	0 ± 0 [0-0]	3,325 ± 1,175 [600-6,050]	38	43:57
IVM-PO-01	920 ± 543 [50-3,000]	1,010 ± 557 [0-3,050]	0 ± 0 [0-0]	1,930 ± 1,098 [50-6,050]	64	48:52
MOX-PO-01	1,590 ± 837 [0-3,700]	2,090 ± 983 [0-5,000]	20 ± 20 [0-100]	3,700 ± 1,818 [0-8,550]	31	44:56
CONTROL-04	1,170 ± 326 [250-2,050]	1,270 ± 339 [350-2,050]	0 ± 0 [0-0]	2,440 ± 649 [850-3,900]	NA	48:52
IVM-INJ-04	1,040 ± 97 [850-1,400]	1,160 ± 73 [1,000-1,350]	0 ± 0 [0-0]	2,200 ± 104 [1,900-2,400]	10	47:53
IVM-PO-04	1,280 ± 270 [750-2,300]	1,430 ± 167 [1,100-1,950]	0 ± 0 [0-0]	2,710 ± 428 [1,850-4,250]	0	47:53
MOX-PO-04	350 ± 183 [0-1,000]	290 ± 97 [100-650]	0 ± 0 [0-0]	640 ± 274 [100-1,650]	74	55:45

In general, higher numbers of female *C. oncophora* were enumerated compared to male *C. oncophora*, with the exception of the group MOX-PO-04, where more male worms were present (55%). Based on the results of a series of proportion tests (Newcombe, 1998), there was no statistically significant difference in the proportion of male and female *C. oncophora* in the treated groups compared to the untreated, control groups. There was also no statistically significant difference found when comparing the groups in receipt of anthelmintic administration ($P > 0.05$).

4.3.2 Comparison of number of eggs *in utero* in female *Cooperia oncophora*

From the 39 calves included in the FEC and worm burden analysis, 36 animals had adult female *C. oncophora* worms present in the 2% small intestinal subsample. The three calves without any female *C. oncophora* in their samples were infected with the FI001 isolate. Two of these had received the IVM pour-on preparation (IVM-PO-01, calves 400949 and 700690), the other, the MOX pour-on preparation (MOX-PO-01, calf 401027). For the remaining animals, the mean number of *C. oncophora* females recovered from the 2% subsample was 30 (± 4 SEM; range 2 - 100). However, as not all enumerated worms were intact, an average of 19 nematodes (± 1.3 SEM; range 2 - 25) were measured for each calf. Figure 22 demonstrates the difference observed between a female *C. oncophora* with a uterus full of eggs, isolated from an untreated control calf, and a female *C.oncophora* worm with an egg-free uterus, seven days after having survived a MOX pour-on application. The results detailing the length of the female worms and eggs *in utero* measurements are displayed in Table 27, together with the arithmetic mean FEC of each group at post-mortem. In total, 679 female *C. oncophora* were examined, with an overall mean length of 12.3 mm (± 0.05 SEM; range 7.6 – 15.4) and a mean numbers of eggs *in utero* of 30 (± 13 SEM; range 0 – 158). There were no significant differences found between the number of eggs enumerated or lengths of nematodes from CONTROL-01 and CONTROL-04 ($P > 0.05$).

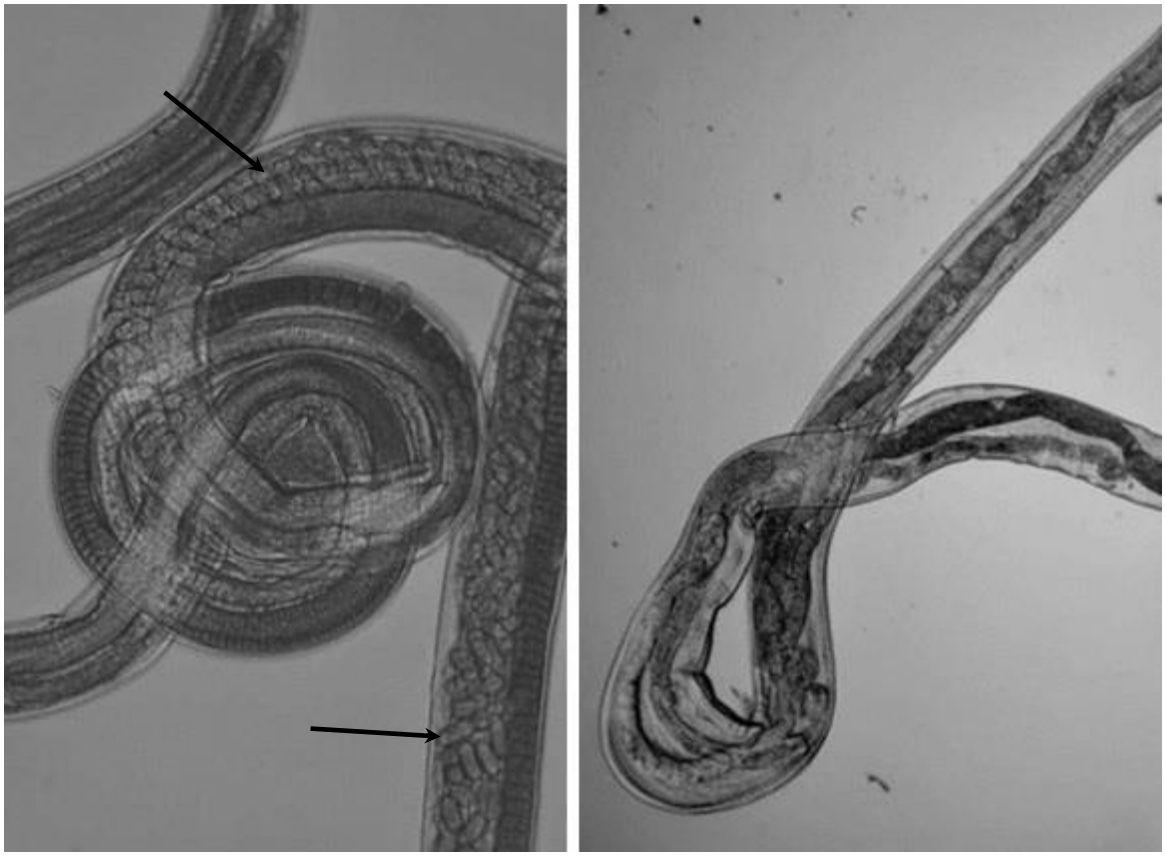

Figure 22: Comparison of differences in numbers of eggs in utero between adult female *C. oncophora*. The image on the left shows an adult *C. oncophora* from a control animal (arrows indicate location of eggs present in utero), whilst the image on the right is *C. oncophora* from a calf treated with MOX pour-on (no eggs observed). Both images were taken under x 100 magnification, following mounting with lactophenol to visualise internal nematode structures.

Table 27: Data from female worm measurements and eggs observed in utero. Data are expressed as arithmetic mean values (± S.E.M) and range of values observed. Groups administered with IVM injectable, IVM pour-on and MOX pour-on applications are referred to as IVM INJ, IVM PO and MOX PO, respectively

Treatment group	N	Arithmetic mean ± SEM [range]		
		Female worm length (mm)	Eggs counted *in utero*	FEC 7 days after administration
CONTROL-01	112	12.5 ± 0.1 [9.2-14.4]	54 ± 3 [0 - 158]	1,491 ± 282 [657-2,844]
IVM INJ-01	79	11.9 ± 0.1 [8.9-14.7]	3 ± 1 [0-72]	30 ± 6 [3-48]
IVM PO-01	66	12.2 ± 0.1 [9.4-14.7]	3 ± 1 [0-34]	13 ± 7 [1-60]
MOX PO-01	82	12.2 ± 0.1 [8.9-15.2]	0 ± 0 [0-0]	4 ± 3 [0-13]
CONTROL-04	88	12.7 ± 0.1 [10.2-15]	61 ± 3 [0-141]	515 ± 166 [135-1,476]
IVM INJ-04	112	12.2 ± 0.1 [6.8-15.4]	23 ± 3 [0-125]	323 ± 28 [216-450]
IVM PO-04	112	12.5 ± 0.1 [7.6-15]	39 ± 3 [0-144]	411 ± 139 [108-1,278]
MOX PO-04	28	11.5 ± 0.2 [10-15]	0 ± 0 [0-0]	1 ± 0.3 [0-3]

The eggs *in utero* data was found to fit a normally distributed error model and so linear regression was used to compare the lengths of examined nematodes from different treatment groups. For the FI001 isolate, there was a significant difference in length found between *C. oncophora* examined from the calves in CONTROL-01 and *C. oncophora* from calves in IVM-INJ-01, IVM-PO-01 and MOX-PO-01 ($P < 0.05$), as displayed in Figure 23. There were no significant differences in worm length between nematodes from calves in IVM-INJ-01 and those from IVM-PO-01 ($P > 0.05$). Nematodes from calves in group MOX-PO-01 were found to be

significantly shorter than both nematodes from calves receiving IVM-INJ-01 and IVM-PO-01 ($P < 0.05$).

For nematodes derived from calves infected with the FI004 isolate, significantly shorter nematodes were found from groups IVM-INJ-04 and MOX-PO-04 than those from the untreated, control group CONTROL-04 ($P < 0.05$). Nematodes recovered from the small intestine of calves in MOX-PO-04 were found to be significantly shorter than those that were examined in samples derived from both IVM-INJ-01 and IVM-PO-01($P < 0.05$).

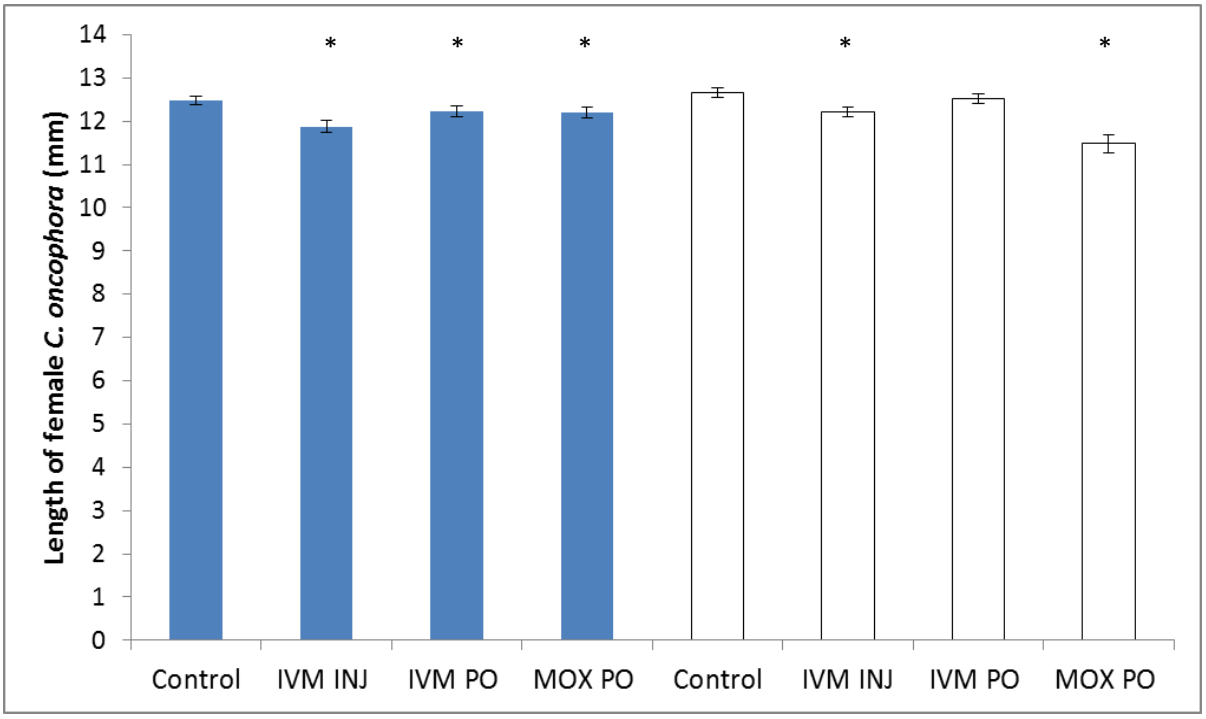

Figure 23: The mean length of female *C. oncophora* nematodes from each anthelmintic group. Blue bars represent FI001, white bars represent FI004, with 'INJ' and 'PO' indicating injectable and pour-on formulations, respectively. Error bars indicate standard error of the mean and asterisks indicate groups with a mean nematode length that is significantly different from that of the respective control group ($P < 0.05$).

Analysis of the number of eggs *in utero* revealed that there were significant differences for both isolates between all treatment groups when each was compared

to the respective untreated control group ($P < 0.05$). No significant difference in egg number was found when comparing IVM-INJ-01 to IVM-PO-01 or when comparing IVM-INJ-04 to IVM-PO-04. However, a statistically significant difference in egg number was found when comparing group MOX-PO-01 to IVM-INJ-01 and IVM-PO-01; a finding also observed when egg numbers from MOX-PO-04 were compared to IVM-INJ-04 and IVM-PO-04 ($P < 0.05$), as displayed in Figure 23.

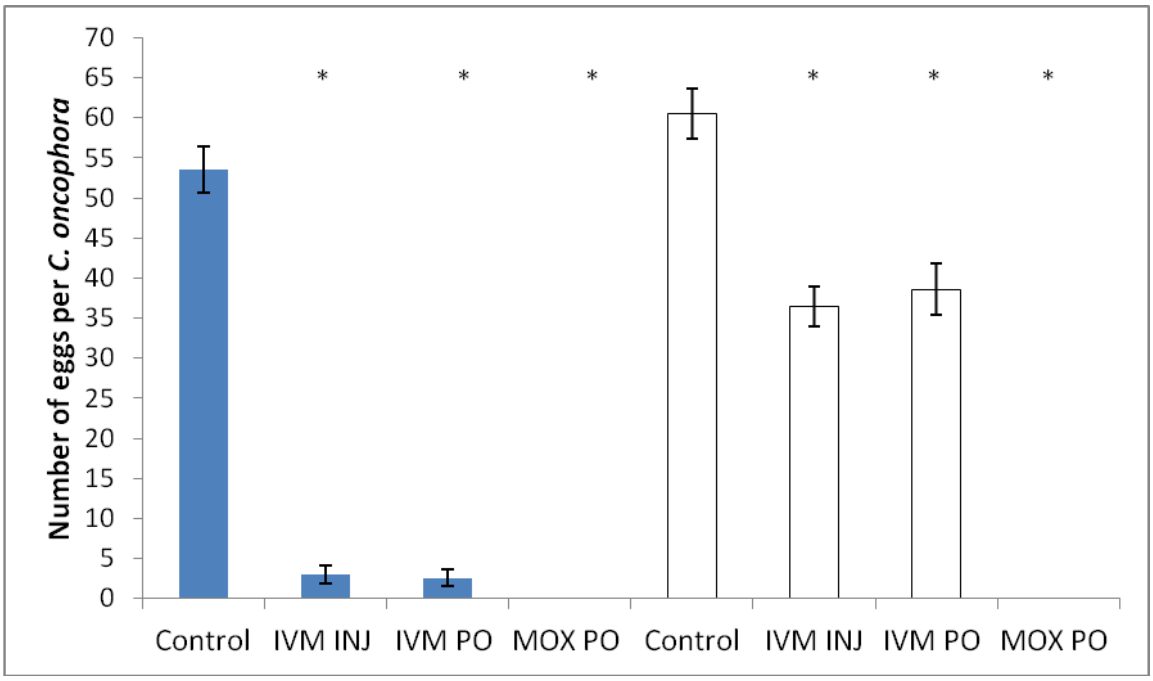

Figure 23: The mean number of eggs counted in utero per female *C. oncophora*, in each of the treatment groups. Blue bars represent FI001, white bars represent FI004, with 'INJ' and 'PO' indicating injectable and pour-on formulations, respectively. Error bars indicate the standard error of the mean and asterisks indicate groups with a mean number of eggs that is significantly different from that of the respective control group (P< 0.05).

Logistic regression performed to analyse the relationship between the length of female *C. oncophora* and the numbers of eggs counted *in utero* per female. For nematodes from CONTROL-01, a statistically significant positive correlation was

observed ($P < 0.05$). For this isolate, statistically significant positive correlations were also observed for data from both IVM-INJ-01 and IVM-PO-01 groups ($P < 0.05$). As no eggs were observed in the uteri of nematodes surviving MOX pour-on administration (MOX-PO-01), this analysis could not be conducted for these nematodes. For nematodes from untreated control calves infected with FI004 (CONTROL-04), a statistically significant positive correlation was observed ($P < 0.05$). This was also observed when the *C. oncophora* from IVM-INJ-04 were assessed ($P < 0.05$). However, a statistically significant relationship was not observed when the data pertaining to the IVM-PO-04 was analysed ($P > 0.05$). As previously stated for FI001, due to the absence of eggs observed *in uteri* in nematodes from MOX-PO-04, this analysis could not be conducted.

4.3.3 Molecular analysis of an area of glutamate-gated chloride channel gene (glc-6) in *Cooperia oncophora*

4.3.3.1 Comparative analysis of *glc-6* nucleotide sequences derived from *C. oncophora* obtained from untreated control calves *versus glc-6* nucleotide sequences obtained from *C. oncophora* harvested from IVM treated calves

Following the phenotypic analyses of adult female *C. oncophora* from isolates FI001 and FI004, differences were seen between worms recovered from the untreated control calves and those nematodes that had survived an anthelmintic administration. To investigate if there were differences present at a genetic level in these groups of parasites, analysis of cDNA encoding for a gene (*glc-6*), for which mutations have been putatively associated with IVM resistance, and which has been upregulated in resistant parasites following anthelmintic exposure (de Graef et al., 2013a) was

conducted. As described in Section 4.2.3.1, RNA was extracted from eight pools of 10 adult nematodes. RNA was extracted from male and female nematodes from untreated control calves infected with FI001 (CON-M-01 and CON-F-01) and from calves infected with FI004 (CON-M-04 and CON-F-04). RNA was also extracted from male and female nematodes surviving IVM injectable administration from FI001 (IVM-M-01 and IVM-F-01) and FI004 (IVM-M-04 and IVM-F-04). For each of the eight pools of RNA, RT-PCR was performed to produce a 319 base pair (bp) cDNA fragment of *glc-6*, located at the 5' end. Ten clones for each amplicon were produced and sequenced along both forward and reverse strands, to yield 20 sequences. These 20 sequences were subsequently aligned to form a consensus sequence for each pool. The consensus sequence was subjected to Blastn analysis to examine percentage identity of each consensus sequence to previously submitted nematode *glc-6* sequences. For each consensus, the sequence results with the three highest percentage identities were recorded. The remaining results from the Blast search were universally less than 40 bp in length and were not derived from parasitic nematodes.

4.3.3.2 Analysis of *glc-6* nucleotide sequence derived from *C. oncophora* from FI001

The consensus from clones amplified from CON-F-01 nematodes showed the highest identity to the full-length *glc-6* sequence for IVM-susceptible *C. oncophora* (Genbank accession number: HF545675) with 100% identity over 319 base pairs (bp). The next closest identity was to the *O. ostertagi glc-6* orthologue (74% identity over 266 bp; HF545676), followed by identity to a messenger RNA sequence for a

putative GluCl subunit (*Hco-glc-6*) in *Haemonchus contortus* (74% identity, 200 over 268 bp; EU006789; (Glendinning et al., 2011). For the consensus representing cDNA from CON-M-01 nematodes, the highest identity was found to the full-length *glc-6* sequence for IVM-susceptible *C. oncophora* (99% identity, 316 over 319 bp; HF545675). The next closest identity was to the *O. ostertagi glc-6* orthologue (74% identity over 266 bp; HF545676), followed by the *Hco-glc-6* orthologue in *H. contortus* (74% identity, 198 over 268 bp; EU006789). The consensus from CON-M-01 and the consensus from CON-F-01 showed similar percentage identities to those observed in the consensus sequence from IVM-M-01 and the consensus from IVM-F-01. For the IVM-F-01 consensus, there was 100% identity to the *C. oncophora glc-6* sequence (HF545675) over 319 bp. This was followed by 75% identity to *Hco-glc-6* (200 bp over 268; EU006789) and 74% identity to *O. ostertagi glc-6* orthologue (74% identity over 266 bp; HF545676). For IVM-M-01 consensus, 99% identity was seen with regard to the *C. oncophora glc-6* sequence (HF545675) with 315 over 319 bp. Identical percentage identities of 74% were seen with *O. ostertagi glc-6* (198 over 266 bp; HF545676) and *Hco-glc-6* (199 bp over 268; EU006789).

4.3.3.3. Analysis of *glc-6* nucleotide sequence derived from *C. oncophora* from FI004

The *glc-6* nucleotide consensus from CON-F-04 showed 99% identity with the IVM susceptible *C. oncophora glc-6* nucleotide sequence (316 over 319 bp; HF545675). The next highest identity was to the *H. contortus Hco-glc-6* orthologue (74% over 268 bp; EU006789), followed by the *glc-6* orthologue from *O. ostertagi* (72% over

310 bp; HF545676). For the *glc-6* consensus derived from CON-M-04, the percentage identity to *C. oncophora glc-6* nucleotide sequence was 100%, over 319 bp (HF545675). The *O. ostertagi glc-6* orthologue had the next highest identity, with 75% identity over 280 bp (HF545676), followed by the *Hco-glc-6* nucleotide sequence of *H. contortus* with 74% identity over 278 bp (EU006789). The consensus from CON-M-04 and the consensus from CON-F-04 showed similar percentage identities to those observed in the consensus sequence from IVM-F-04 and the consensus from IVM-M-04. The *glc-6* consensus generated from amplifying cDNA from IVM-F-04, showed 100% identity to *C. oncophora glc-6* over 319 bp (HF545675). The second highest identity was with *H. contortus Hco-glc-6* (75% over 268 bp; EU006789), followed by *O.ostertagi glc-6* (74% over 266 bp; HF545676). When the *glc-6* nucleotide consensus from cDNA generated from IVM-M-04 examined, a 99% identity was found for the *C. oncophora glc-6* nucleotide sequence (317 over 319 bp; HF545675). Identities of 74% were observed for *H. contortus Hco-glc-6* (199 over 268 bp; EU006789) and *O.ostertagi glc-6* (196 over 266 bp; HF545676).

4.3.3.4 Comparative amino acid sequence analysis of *glc-6* in *C. oncophora* generated from untreated control calves and *C. oncophora* surviving injectable IVM administration

Each nucleotide consensus was translated to a peptide sequence and aligned in Clustal Omega, as displayed in Figure 24. Pairwise alignments were conducted to test for similarity between consensus, as reported in Table 28. For comparative

purposes, pairwise alignments also included *glc-6* amino acid sequences derived from an IVM-susceptible *C. oncophora* isolate (HF545675; de Graef et al., 2013a), a non-related IVM-resistant *C. oncophora* isolate (unpublished data (de Graef et al., 2013a)) and an IVM-susceptible *O. ostertagi* isolate (HF545676; de Graef et al., 2013a), all originating in Belgium.

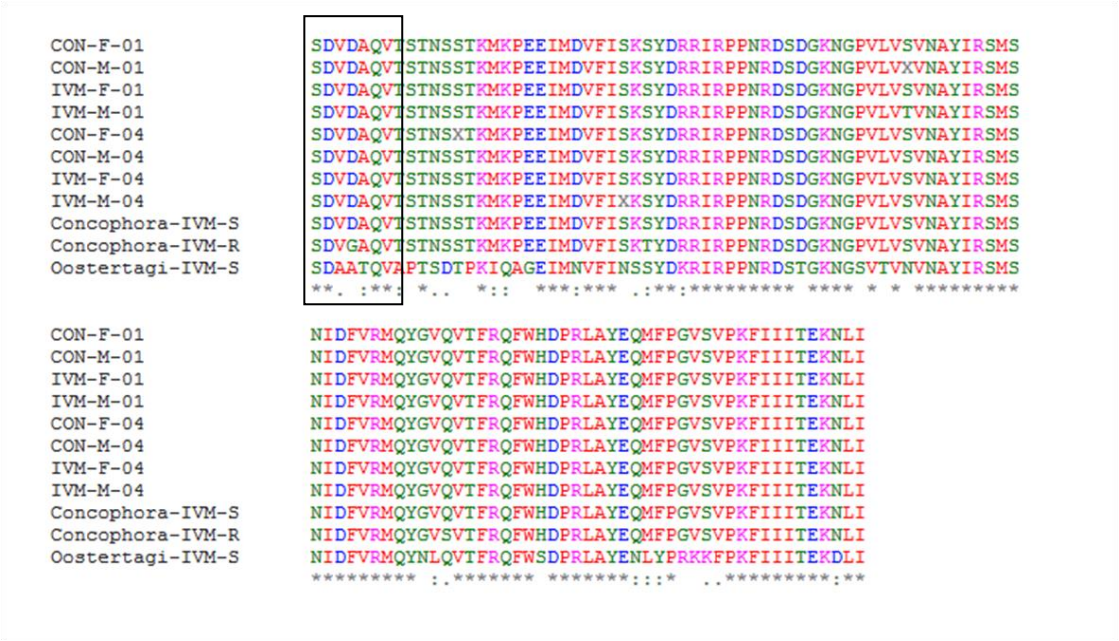

Figure 24: Peptide sequences of the glc-6 gene, generated from nucleotide consensus data from, derived from 20 sequences from C. oncophora from two isolates, having being exposed, or not, to IVM in vivo. Unexposed nematodes and those surviving IVM are denoted by "CON" and "IVM" respectively. Male and female nematodes are denoted by "M" and "F" and the isolate from which the nematodes originated is denoted by 01 or 04. For comparison purposes, peptide sequences derived from an IVM-resistant C. oncophora and IVM-susceptible C. oncophora and O. ostertagi isolates are also included (De Graef et al., 2012). The region of peptide suggested to show differences between resistant and susceptible C. oncophora isolates (De Graef et al., 2013a) is highlighted within the box.

In total, four differences in peptide sequence were noted, compared to the IVM-susceptible *C. oncophora* (HF545675) peptide sequence. For CON-M-01, at position 50 on the peptide sequence, threonine (ACC) replaced serine (ACT) on six occasions within the 20 sequences. At the same location, for IVM-M-01, threonine (ACT) replaced serine (ACT) on six occasions. For isolate FI004, at location 11 on the peptide sequence for CON-F-04, tryptophan (TGG) and threonine (ACG) both replaced serine (TCG) on eight occasions. At position 25 on the peptide sequence, for IVM-M-04, serine (AGC) was replaced by a cysteine residue (TGC) on 12 occasions and by arginine (CGC) on eight occasions. Similar differences in peptide sequence were not observed in IVM-resistant or susceptible *C. oncophora* isolates examined in the Belgian study (de Graef et al., 2012), where a difference in sequence between IVM-R and IVM-S isolates was seen at the N terminal end of the G:LC-6 peptide sequence (indicated by the box in Figure 24).

As expected a substantial degree of identity was observed among the GLC-6 peptide sequences derived from FI001 and FI004 and that of the IVM-susceptible *C. oncophora* isolate, with percentage identity > 99% for all comparisons. Percentage identity of the FI001 and FI004 peptide sequences compared the IVM-R *C. oncophora* isolate was >96% (Table 28). For almost all isolates, including the IVM-susceptible *C. oncophora* isolate (HF545675), the percentage identity was 69.81% to IVM-susceptible *O. ostertagi glc-6* sequence (HF545676). A percentage identity of 70.1% was observed between the IVM-R *C. oncophora* isolate and the IVM-susceptible *O. ostertagi glc-6* sequence.

Table 28: Pairwise comparison analysis, presented as percentage identity, of peptide sequences derived from *C. oncophora* from two isolates, having being exposed, or unexposed, to IVM *in vivo*. Unexposed nematodes and those surviving IVM are denoted by "CON" and "IVM", respectively, with male (M) and female (F) nematodes from field isolates 001 (01) and 004 (04) denoted accordingly. For comparison with other isolates, IVM-susceptible *C. oncophora* (C.o (S)), IVM-resistant *C. oncophora* (C.o (R)) and *O. ostertagi* (O.o) isolates are also included (De Graef et al 2012).

	CON-F-01	CON-M-01	IVM-F-01	IVM-M-01	CON-F-04	CON-M-04	IVM-F-04	IVM-M-04	C. o (S)	C. o (R)	O. o
CON-F-01		99.06	100	99.06	99.06	100	100	99.06	100	97.2	69.81
CON-M-01			99.06	99.06	98.11	99.06	99.06	98.11	99.06	96.3	69.81
IVM-F-01				99.06	99.06	100	100	99.06	100	97.2	69.81
IVM-M-01					98.11	99.06	99.06	98.11	99.06	96.3	69.81
CON-F-04						99.06	99.06	98.11	99.06	97.2	69.81
CON-M-04							100	99.06	100	97.2	69.81
IVM-F-04								99.06	100	97.2	69.81
IVM-M-04									99.06	96.3	69.81
C. o (S)										97.2	69.81
C. o (R)											70.1
O. o											

4.4 Discussion

The aims of the CET were to ascertain if the findings of IVM resistant *Cooperia* spp. from the FECRTs conducted in Chapter 3 were reproducible under controlled conditions and to investigate the efficacy of an alternative application method (namely, a topical pour-on) and anthelmintic product (MOX). The results have proven that both field isolates FI001 and FI004 contained *C. oncophora* nematodes that are resistant to the effects of IVM and MOX. The CET in this study was conducted as far as possible in accordance with the recommended WAAVP guidelines (Wood et al., 1995). However, from the results displayed here, there are clear differences displayed in the plasma, FEC and worm burden analyses from different treatment groups and between the two isolates. Blood plasma was taken during the CET for two reasons. First, to confirm that animals administered with anthelmintic had the product circulating in their bloodstream and second, to examine the differences in plasma levels between the anthelmintic classes and application methods, as anthelmintic levels in blood plasma have been associated with clinical efficacy (Lanusse et al., 1997). Here, peak IVM and MOX plasma concentrations observed fell within previously published concentration ranges (Gayrard et al., 1999; Lifschitz et al., 1999a; Lifschitz et al., 1999b; Sallovitz et al., 2002). The pharmacological pattern from MOX-PO-01 and MOX-PO-04 groups were very similar and were far lower than the plasma concentrations observed with IVM-PO-01 and IVM-PO-04. This result is to be expected, given that MOX is believed to have a faster clearance time from the plasma to adipose tissue, which is linked to longer persistence of the molecule within the host (Lanusse et al., 1997; Bousquet-Mélou et al., 2004). The finding of lower IVM concentrations in the plasma from calves

administered with a pour-on IVM application, particularly in IVM-PO-01, is also to be expected. For IVM pour-on formulations, whilst they have been shown to be efficacious against IVM-susceptible *O. ostertagi* and *C. oncophora* (Herd et al., 1996; Gayrard et al., 1999), the IVM is less easily absorbed through the skin than it is from a subcutaneous injection site (Bousquet-Mélou et al., 2004; Prichard et al., 2012). The observation that no IVM was found in plasma samples from one calf after it was administered with the injectable formulation of IVM was unexpected. The calf was believed to have been administered correctly and all calves were injected by the same operator. Consequently, there were only four animals in this treatment group, which increases the risk of a Type II error (not rejecting the null hypothesis when an effect is present, but not detected in the analysis). Collecting samples of blood plasma samples to determine anthelmintic concentrations is not a practice routinely described in publications reporting CET results, nor stipulated in the current WAAVP guidelines (Wood et al., 1995). However, in situations such as confirming anthelmintic efficacy against a suspected resistant isolate, the findings from this CET suggest the use of plasma analysis should be considered to confirm that anthelmintic was received by all animals. Failure to do so may lead to uncertainty regarding the results, as results cannot be differentiated between a case of anthelmintic misadministration, and one of 'true' anthelmintic resistance. The comparison of plasma concentrations between published studies can be problematic as, even under controlled conditions, differences in pharmacokinetic data can be seen due to factors such as animal breed (Sallovitz et al., 2002), gender (Ndong et al., 2007), age and diet quantity, quality and composition (Sanyal et al., 1995), as well as study design, i.e. different sampling periods, anthelmintic applications used and non-

standardised methods of analysis (Lanusse et al., 1997). Previous studies have also reported pharmacokinetic differences between male and female cattle (Lo et al., 1985; Toutain et al., 1997) and differences have been reported among generic IVM products (Lifschitz et al., 2004). In the latter study, comparison of four generic IVM products, following subcutaneous administration in cattle, displayed differences in time to reach peak IVM concentration in plasma (range: 1.1 to 4.3 days), with a difference of 11 ng ml^{-1} between mean peak IVM concentrations (range: 22 to 33 ng ml^{-1} IVM) (Lifschitz et al., 2004). For this reason, here, the anthelmintic products that were used contained the original compounds, namely IVOMEC® (Merial Animal Health) and Cydectin® (Pfizer Animal Health). Another common issue is the use of different cattle breeds, which may have inherent differences in metabolism or body composition, a particular importance with ML compounds as both IVM and MOX are lipophilic and are distributed within the body fat reserves (Bassissi et al., 2004). This means that data collected by one study, for example the use of 400 kg dairy cattle (Herd et al., 1996) may be different to one which uses 200 kg Hereford cattle (Lanusse et al., 1997). A previous study designed to examine the effect of cattle breed on pharmacokinetic data was conducted by comparing Aberdeen Angus and Holstein cattle, where differences in C_{max} values and time to reach C_{max} following MOX pour-on administration were found. C_{max} values of 2.33 ± 0.28 ng ml^{-1} and 5.08 ± 0.94 ng ml^{-1} were observed for Holstein and Aberdeen Angus cattle, respectively (Sallovitz et al., 2002). Time to reach the C_{max} value also differed between groups, with peak seen later with the Aberdeen Angus cattle (five days following administration) than with the Holstein cattle (two days following administration; (Sallovitz et al., 2002)). Here, this potential issue was avoided by

using male dairy calves that were balanced for age and weight, and no statistically significant correlations were found when ML plasma concentrations were compared to age or weight of calves.

From the FEC analysis conducted on day of anthelmintic administration, differences in FEC were observed between calves infected with FI001 and those infected with FI004. For the calves infected with FI001, a mean of 1,008 EPG was observed, in contrast to the mean FEC of 613 EPG for calves infected with FI004. This can be attributed to a greater proportion of *O. ostertagi* L_3 present in FI004 (40%), compared to 20% *O. ostertagi* present in FI001. *O. ostertagi* is known to be less fecund than *C. oncophora*, containing, on average, 30 eggs per uterus at any one time, compared to an average of 60 eggs in the uterus of a *C. oncophora* female (Kloosterman, 1971). As all calves received approximately the same number of larvae, the difference in genus proportions between the two isolates could account for the higher FECs observed in calves infected with FI001 prior to anthelmintic administration, compared to the FEC data from calves infected with FI004. Although daily fluctuations in FEC were seen with both CONTROL-01 and CONTROL-04 groups, these differences were not statistically significant, and FECs were far higher than the FECs observed under the natural infection conditions of the on-farm FECRT, where the mean day of treatment FECs were 74 and 86 EPG for FI001 and FI004, respectively.

The change in FEC after anthelmintic application is negatively correlated with the increase in ML plasma concentrations, across all treatment groups. Based solely on reduction in FEC, results from groups IVM-INJ-01, IVM-PO-01, MOX-PO-01 and

MOX-PO-04, would appear to indicate a high level of efficacy. If taken in isolation from the rest of the results presented here, an erroneous assumption of ML sensitivity may have been made. However, it must be noted that these FECs were not taken 14 days after anthelmintic administration, as indicated by FECRT guidelines for ML products (Coles et al., 1992). IVM has been previously shown to have a temporary, paralysing effect on nematode uterine musculature, as demonstrated by work with the ovine nematodes *Trichostrongylus colubriformis* (Bottjer and Bone, 1985) and *Cooperia curticei* (McKellar et al., 1988). The FEC data from groups IVM-INJ-04 and IVM-PO-04 suggests that after sufficient time has elapsed, egg-laying can resume in resistant isolates, adding further credence to appropriate sampling times being adhered to when conducting FECRTs (Coles et al., 1992).

Analysis of adult nematodes recovered at necropsy confirmed the presence of IVM and MOX resistant *C. oncophora* in both isolates, FI001 and FI004. Conversely, IVM and MOX were found to be highly effective against *O. ostertagi* in both isolates, with percentage reductions in abomasal worm burdens in excess of 99%. Percentage reductions in adult *C. oncophora* burdens were 38%, 64% and 31% for groups IVM-INJ-01, IVM-PO-01 and MOX-PO-01, respectively, when compared to the numbers present in samples from CONTROL-01. Similarly, percentage reductions in adult *C. oncophora* were 10%, 0% and 74% for groups IVM-INJ-04, IVM-PO-04 and MOX-PO-04, respectively, compared to *C. oncophora* present in samples from CONTROL-04. The findings of IVM inefficacy confirm the suspected resistance in *Cooperia* spp. detected with the on-farm FECRTs described in Chapter 3 (McArthur et al., 2011). The discovery that these isolates are also MOX-resistant

means these are the first confirmed cases of MOX-resistant cattle nematodes in the UK (Bartley et al., 2012). The presence of ML-resistant *C. oncophora* surviving anthelmintic administration is not surprising, as this species has globally been implicated in almost all initial reports of AR in cattle and was identified during pre-registration efficacy trials to be one of the dose-limiting helminth species for both IVM (Egerton 1979, Egerton et al 1981) and MOX (Scholl et al 1992). Higher concentrations of both products were required to match efficacy obtained against adult nematodes of abomasal species such as *O. ostertagi* (Egerton et al 1981, Scholl et al 1992). Although IVM resistance was confirmed in *C. oncophora* in both field isolates and observed when using IVM injectable and pour-on preparations, there are differences in observed efficacy levels, based on adult small intestinal worm burdens. With group IVM-PO-01, a higher percentage efficacy (64%) was observed than in group IVM-INJ-01 (38%), despite a higher C_{max} value being obtained in plasma collected from calves in group IVM-INJ-01. This is unexpected, as it is generally accepted that application of anthelmintics by subcutaneous injection is a more efficient route in cattle, allowing greater and more immediate bioavailability when compared to pour-on or oral applications (Gayrard et al., 1999; Laffont et al., 2001). Conversely, with groups IVM-INJ-04 and IVM-PO-04, a higher C_{max} value was observed with IVM-PO-04 compared to IVM-INJ-04, but a zero percent reduction in *C. oncophora* was recorded in group IVM-PO-04, compared to 10% reduction seen with group IVM-INJ-04. These findings may indicate that whilst pharmacokinetic data from ML administration may appear to reflect FEC data, it may not reflect the extent of the effect on adult nematode burden at 7 days post treatment.

Application with the MOX pour-on in group MOX-PO-04 produced a greater reduction in small intestinal worm burden (74%) than that observed with either of the IVM applications (0% and 10% for IVM-PO-04 and IVM-INJ-04, respectively). However, with FI001, the lowest reduction in *C. oncophora* numbers was observed in calves administered with MOX (31%), compared with reductions observed with IVM-INJ-01 (38% reduction) and IVM-PO-01 (64% reduction). It might have been expected that MOX would have been more effective in reducing small intestinal worm burdens than IVM, as it is believed to be more lipophilic and have a higher potency compared to IVM (Kieran, 1994). The results from work conducted by Ranjan et al (1992) suggested MOX exerts selection pressure on female *C. oncophora*, as greater efficacy was achieved against female *C. oncophora* than adult male *C. oncophora*, with efficacies of 98.4% and 93.8% respectively. The results are in contrast to those of the current study where the ratio of male to female *C. oncophora* was more biased to females (55% of population). Our results correlate with previous work conducted examining two *C. oncophora* isolates unexposed to anthelmintics, where the average percentage of male nematodes was 47%, based on results from 10 calves (Bairden et al., 1992). The one exception in this study was with group MOX-PO-04, where 10% more male *C. oncophora* were found than female (55% and 45%, respectively). This result indicates that, as with Bairden et al., (1992), sex ratios may differ between isolates and anthelmintic selection may not be wholly responsible for any observed differences and may not be true for all *C. oncophora* isolates.

Efficacy based on comparison to control animal data clearly shows resistance to both IVM and MOX, regardless of application method, in both isolates. As the controlled conditions of the CET restrict a number of confounding issues, discussed above, such as use of calves of a similar breed and age, reasons for differing efficacies could be attributable to the previous use of anthelmintics on Farms 001 and 004, prior to isolation of these isolates. Data regarding parasite management regimes was collected (as described in Chapter 2) and revealed both farms generally employed similar practices. First season grazing calves were administered with anthelmintics twice (Farm 001) or three times (Farm 004) per year. Both farms had administered ML products for the previous five years and had used pour-on products. Farm 001 volunteered the information that eprinomectin had been used in the preceding year and other ML products were used prior to this. Differences were observed in administration practices; as while Farm 004 administered anthelmintics to all calves and individually weighed animals to determine dose volumes, Farm 001 usually estimated the weight of the calves to determine dose rates. While Farm 001 was a cattle-only enterprise, Farm 004 also farmed sheep, which were grazed with cattle. Depending on the anthelmintic regime employed with the sheep, additional selection pressure for ML resistant nematodes may have been provided, especially in *Cooperia* spp., which are proven to infect sheep, as well as cattle (Keith, 1953; Isentstein and Porter, 1964).

The mean lengths of the female *C. oncophora* reported here are longer than those published previously, ranging between 11.5 – 12.7 mm. Work conducted in 1963 found female *C. oncophora* to measure on average 9.43 mm (Isentstein, 1963) and

veterinary textbooks commonly cite *C. oncophora* as being of similar size to *O. ostertagi* (Urquhart et al., 1996) or measuring 6 – 8 mm (Taylor et al., 2002). In other published literature, however, mean measurements of adult *C. oncophora* females ranged from 8.32 - 11.45 mm (Smith 1970), 9.3 - 11.10 mm (Bairden et al., 1992), and 11.5 - 13.3 mm (Albers et al., 1982). In this study, the results relate most closely to that reported by Albers et al (1982), who reported a statistically significant correlation between female *C. oncophora* mean length and the time of calf necropsy following infection. Nematodes recovered at 55 days PI were significantly larger than those recovered at 28 days PI, measuring on average 13.3 and 11.8 mm, respectively ($P < 0.010$) (Albers et al., 1982). Conversely, the opposite was observed in *O. ostertagi* worm length, where a decrease of 7 – 8 % in female length was detected over time (Michel et al., 1978b). These findings may go some way to explaining why the measurements of *C. oncophora,* not exposed anthelmintic administration, are so variable between studies. The two isolates characterised here also appear to show differences in nematode length, depending on the anthelmintic administered. Outwith *C. oncophora* from CONTROL-01 and CONTROL-04, the shortest nematodes were seen in groups IVM-INJ-01 (mean 11.9 mm) and MOX-PO-04 (mean 11.5 mm). It has been previously hypothesised with the ovine nematode *Teladorsagia circumcincta,* that nematodes resistant to levamisole were larger and more fecund than susceptible nematodes (Leignel and Cabaret, 2001). As with the small intestinal worm burden data, the differences observed between isolates, may be a result of prior anthelmintic selection pressure, resulting in different responses in the different treatment groups.

Whilst lengths of *C. oncophora* worms appear to be variable between studies, so too are reports of the numbers of eggs observed *in utero*, potentially attributable to a number of factors, such as provenance of *C. oncophora* isolate, host immunity and that the production of nematode eggs is not known to be a continuous process (Kloosterman, 1971; Albers et al., 1982; Kloosterman et al., 1984; Bairden et al., 1992). In one previous study examining the effect of isolate pathogenicity in four groups of three month-old calves infected with mixed isolates of *C. oncophora* and *O. ostertagi*, 10 female *C. oncophora* per group were examined and an average of 25, 37, 40 and 46 eggs per nematode were found (Bairden et al., 1992). Differences in the numbers of eggs per *C. oncophora* female have also been found to differ dependent on the age of calf infected, with average numbers of eggs found in *C. oncophora* infecting yearling cattle to be less than half of that found in *C. oncophora* from first season calves, with a mean of 30 and 78 eggs recorded, respectively (Smith, 1970). Here, in female *C. oncophora* from groups CONTROL-01 and CONTROL-4, an average of 54 and 61 eggs were counted per nematode. This finding is similar to a recent CET in Belgium, where female *C. oncophora* from untreated control calves were found to contain an average of 67 eggs per nematode (de Graef et al., 2012), with nematodes surviving 14 days after injectable IVM or MOX administration found to have an average of 57 and 38 eggs per nematode, respectively (de Graef et al., 2012). Overall, the findings from the analysis of the numbers of eggs in utero show that eggs present in the examined female nematodes from the control groups can reflect the FEC observed on day of necropsy, a finding which was also observed in the Belgian CET, 14 days after anthelmintic administration (de Graef et al., 2012). Where this becomes more complex is when

ML administrations alter egg generation or oviposition in worms. The low numbers of eggs present in *C. oncophora* from groups receiving IVM or MOX administration would indicate that egg production had been suppressed. It has been previously shown that in nematodes surviving therapeutic levels of MLs, the musculature of the uterus may be paralysed (McKellar et al., 1988; Scott et al., 1991; McKenna, 1997) which may result in egg production resuming after ML levels have dropped. The fact that eggs were observed in the on-farm FECRT 14 days after IVM administration, as presented in Chapter 3 (McArthur et al., 2011), would indicate this is a likely scenario for these two *C. oncophora* isolates.

The molecular analysis of the *glc-6* gene fragment previously shown to contain mutations that were putatively associated with ML resistance (de Graef et al., 2012), did not reveal any of the same polymorpisms here that led to amino acid differences at the N terminus. As a result, it does not appear that differences in this region of peptide sequence for the *glc-6* gene are responsible for the ML resistance observed in the *C. oncophora* isolates studied here, (FI001 and FI004). This is further compounded by the high levels of percentage identity (in excess of 99%) between the IVM-susceptible *C. oncophora* isolate from Belgium and the two IVM-resistant *C. oncophora* isolates from this CET. This may mean that there are other mutations present on different subunit genes that encode the proteins that make up the GluCl channel (Wolstenholme, 2011). As a result, for each subunit within the GluCl channel, there are many potential areas for mutations to arise that may affect the conformation of the pore, and limiting the potential for MLs to bind (Hibbs and Gouaux, 2011). Furthermore, it seems increasing more likely that ML resistance in

ruminant nematodes has a multigenic origin, with multiple mechanisms involved. MLs are also known to act on receptors such as GABA (Prichard, 2007) and the dopamine-gated chloride channel (Rao et al., 2009). In addition, considerable evidence for the involvement of ATP-binding-cassette transporters, such as the P-glycoproteins, interfering with ML-transport, has been published for sheep nematodes, such as *T. circumcincta* (Dicker et al., 2011). Overall, the genetic basis of ML resistance appears to be highly complex, and is unlikely to be attributable to a single molecular marker. It so leads that whilst the mutation in *glc-6* may confer some conformational abnormalities in the GluCl channel of a specific *C. oncophora* isolate, such as may have occurred in the IVM-resistant isolate from Belgium, it may not be present in all isolates, as the mutation does not appear to be present in either of the isolates studied here.

In this chapter, the aims were to characterise two field isolates of nematodes, which were believed to be resistant to IVM administration. The results from the CET confirmed that both isolates contain not only IVM-resistant *C. oncophora*, but also MOX-resistant *C. oncophora*. Phenotypic analysis of the surviving female nematodes revealed differences in worm length and oviposition between treatment groups and between isolates, whereas genotypic analysis of a gene fragment previously associated with ML resistance in *C. oncophora* (de Graef et al., 2013a) revealed that the same single nucleotide polymorphism was not identified in the IVM/MOX resistant isolates here. Given the time and labour costs associated with characterising these isolates, and the complicated nature of ML resistance and the lack of molecular markers for detection, it is imperative that other methods of

diagnosing resistance are developed. For example, *in vitro* assays could be validated for use between laboratories and could provide rapid, reliable screening tests for resistant, and potentially resistant field isolates of nematodes. This was the next objective of this work, which shall be described in the following chapter (Chapter 5), towards developing an optimised *in vitro* test for the detection of ivermectin resistance in mixed isolates of gastrointestinal nematodes.

Chapter 5: Evaluation of the Larval Migration Inhibition Test (LMIT) for use with mixed species cattle nematode isolates

5.1 Introduction

For field populations of parasitic nematodes, characterisation of macrocyclic lactone (ML) sensitivity currently relies on the use of *in vivo* tests, such as the controlled efficacy test (CET) and the faecal egg count reduction test (FECRT) (discussed in Chapters 3 and 4). Clear guidelines for conducting FECRTs in cattle remain to be established and currently researchers follow guidelines primarily described for small ruminants. In addition, faecal egg counts (FEC) in cattle populations are often low compared to those found in sheep and goats (for example, less than 100 eggs per gram (EPG)). By basing tests on FEC alone, inaccurate evaluations of sensitivity are possible as considerable differences in efficacy can be observed due to slight changes in FEC (Boersema, 1983; Sutherland and Leathwick, 2011). It must also be taken into account that faecal samples from naturally infected calves on farms are likely to contain eggs of more than one nematode species, which may inherently differ in response to anthelmintic treatment. For example, there may be direct effects on adult nematodes such as suppression in egg production following anthelmintic administration, as previously found in nematode species such as *Cooperia curticei* (McKellar et al., 1988) and *Trichostrongylus colubriformis* (Bottjer and Bone, 1985), or indirect effects, such as those on female egg output responding to changes in adult worm density (Keymer, 1982)

Current *in vivo* methods for the detection of anthelmintic resistance have a number of issues, as previously described in Chapters 3 and 4. The FECRT has been demonstrated to lack sensitivity in detecting emerging resistance; studies with ovine nematode isolates demonstrated that this test was unable to detect benzimidazole resistance at levels below 25% with *Teladorsagia (Ostertagia)* spp. and *Trichostrongylus colubriformis* (Martin et al., 1989). These results were achieved through combining different proportions of larvae from isolates known to be either resistant or susceptible to benzimidazoles in ratios of 90:10, 75:25, 50:50, 25:75 and 90:10, with susceptible and resistant larvae, respectively. Lambs were experimentally infected with the isolate mixes and FECRTs conducted. Resistance proportions of between 25% and 50% were required for an unequivocal declaration of resistance in both species using the FECRT (Martin et al., 1989). The alternative *in vivo* test is the CET, but despite being the current gold standard for determining anthelmintic resistance in ruminant nematodes, it requires the use of experimental animals and is labour intensive (Coles et al., 2006). Consequently, there is a clear need for simple robust diagnostic tests that can be performed across laboratories (Kotze et al., 2006).

As there are currently three classes of anthelmintic licensed for use in cattle and identification of emerging resistance to a particular class will be beneficial for informing future control strategies based on best practice principles. As demonstrated in Chapter 2, ML anthelmintics appear to be the most popular class administered to cattle in the UK, so the ability to detect emerging resistance to this class is of particular interest. The availability of a technically straightforward and

robust *in vitro* diagnostic test able to identify relatively small quantitative changes in dose response to anthelmintics would be extremely useful (Martin and Le Jambre, 1979). The use of *in vitro* tests as a means to observe anthelmintic activity on parasitic nematodes has been reported for some time (Lamson and Brown, 1936). Early studies examined the effect of anthelmintics on adult nematodes collected at post-mortem, with the caveat that the worms were outside the host in an environment not normally experienced by adult stages and were in the process of dying (Leiper, 1963, cited in Leland Jr, et al., 1971). The first *in vitro* test to utilise *Cooperia punctata* third stage larvae (L_3) was published in 1971 and examined the extent of entangling exhibited by *C. punctata* L_3 when incubated with organophosphates (OP) (Leland Jr. et al., 1971). Two tests were described: a presumptive and a confirmative test to identify a range of OP concentrations likely to kill 50% of L_3. The confirmatory test procedure was lengthy and required the L_3 to be placed in OP medium, then observed at hourly intervals for 6 h, followed by daily examinations for a week. The L_3 were then scored by an observer who categorised L_3 as belonging to one of four "dead or alive" categories (namely, 100% dead, >90% dead, >50% dead and <50% dead). Live L_3 from the latter three categories were subsequently scored as: sluggish; active, but not entangled; active and entangled. The ratings were then used to form the basis of a presumptive LD50 concentration range; i.e. the concentration estimated to kill 50% of L_3. The confirmatory test was similar to the presumptive test; however, numbers of live and dead L_3were counted and used to generate LD50 estimates, rather than a rating scheme (Leland Jr. et al., 1971). This protocol was then used to measure the effectiveness of anthelmintics such as levamisole (LEV), pyrantel (PYR) and thiabendazole (TBZ) (Leland Jr. et al., 1975).

The presumptive test was deemed to be relatively rapid, in that observations of only a few seconds were required to make an evaluation. The tests were expanded to evaluate if TBZ paralysis could be reversed by incubation in TBZ-free media, but the LD50 range was found to be unaltered, suggesting TBZ induced paralysis was not reversible (Leland Jr. et al., 1975). However, this finding was challenged in later studies (Boersema, 1983) and paralysis was found to be reversible. Further studies evaluated tests that measured the effects of anthelmintics on larval motility (Martin and Le Jambre, 1979). In these studies, *Teladorsagia* spp. L_3 were incubated in a range of LEV concentrations for 24 h, followed by a period of observation during which larval motility was scored by an observer as either "normal" (seen to be moving) or "paralysed" (no movement during a five second observation). The percentage of paralysed L_3 were subjected to probit analysis and plotted as a dose response curve (Le Jambre et al., 1976). The L_3 exhibited a spastic paralysis which was found to be reversible (Martin and Le Jambre, 1979), particularly at concentrations of between 400 – 1,000 µg ml^{-1} (Geerts et al., 1987), which lead to claims that the test was unrepeatable (Boersema, 1983). Geerts et al (1987, 1989) repeated this test with L_3 from a field isolate of *O. ostertagi* believed to be resistant to LEV and morantel tartrate (MOR), but found the method to be too subjective and insensitive in detecting MOR resistance, which had been confirmed previously by CET. Attempts to optimise the test further with the addition of 25 µm sieves to separate migrating worms produced inconsistent results; LD50 estimates varied within isolates and larvae from resistant isolates often exhibited lower LD50 estimates than those from susceptible ones (Sangster et al., 1988). A later study also concluded that this test was unsuitable for testing TBZ resistance as the action of

TBZ does not directly cause muscular paralysis of larvae and so it was deemed unlikely that sufficient paralysis could be consistently achieved for the purposes of the test (Sutherland and Lee, 1990).

Bennett and Pax (1986) surmising that a reduction in larval motility is the action of most anthelmintics, devised a micromotility meter to objectively measure the effect of ivermectin (IVM) against L_3 of *Ascaris suum*, *Caenorhabditis elegans* and *Nippostrongylus brasiliensis*. Unlike LEV-treated L_3, IVM treated L_3 exhibit a flaccid paralysis, primarily at the midgut region, which extends throughout the body (Geary et al., 1993; Kotze et al., 2012). The L_3 were incubated for up to 1 week in IVM at concentrations ranging from 10^{-8} to 10^{-6} M, and tested at 24 h intervals in the motility meter. A motility index was produced dependent on the rate of movement perceived in one of three motility channels, connected to a photodiode and microprocessor. A constant signal from the photodiode (i.e. not interrupted by moving L_3) would give a motility index of zero. This test was the first to produce quantitative data regarding larval motility (Bennett and Pax, 1986). The meter was then used to examine the effect of IVM, LEV and albendazole on *Haemonchus contortus* L_3, and a significant reduction in motility was reported over concentrations of 10 - 100 µg ml^{-1} with all anthelmintics tested (Folz et al., 1987). However, the authors conceded there was a major limitation in the use of the micromotility meter, in that the test was unable to differentiate between dead L_3, paralysed L_3 or L_3 displaying abnormal (non-sinusoidal) movement (Gill et al., 1991). Additionally, significant differences were found between the three channels forming the meter and

so a strict randomisation process needed to be followed to avoid confounding results (Folz et al., 1987).

Douch et al. (1983) replaced anthelmintics with abomasal mucus to determine immunomodulatory effects on migration of a variety of ovine nematode species (namely, *H. contortus, Nematodirus spathiger, T. circumcincta* and *T. colubriformis*). Here, the L_3 were exsheathed and incubated in mucus at 37°C for 3 h. Next, they were transferred to a migration apparatus consisting of agar blocks and a nylon mesh with pore size of 100 µm. Larvae were left to migrate overnight and aliquots of migrated and non-migrated L_3 counted to calculate mean percentage migration and subsequently, LD50 estimates calculated (Douch et al., 1983). A similar method was also used to study migration of *O. ostertagi* and *C. oncophora* L_3 following incubation with abomasal or small intestinal mucus, respectively (Kimambo and MacRae, 1988). Douch and Morum (1994) later developed this method for use with anthelmintics (IVM, LEV, MOR and TBZ) using a lower volume of agar to test a variety of ovine nematode isolates. IVM, LEV and MOR were found to significantly inhibit larval migration over concentrations of 1 - 10 µg ml^{-1}, and migration was inhibited at TBZ concentrations in excess of 10 µg ml^{-1} (Douch and Morum, 1994). Sigmoidal dose response curves were generated by exposing *T. colubriformis* to a range of IVM, LEV and MOR concentrations, whereas migration following exposure to TBZ was erratic, attributed in part to the insolubility of TBZ. This test was subsequently adopted to examine IVM resistance in *H. contortus* and identified significant differences in larval migration across a range of IVM resistant and susceptible isolates (d'Assonville et al., 1996). Wagland

et al., (1992) sought to simplify the protocol published by Douch et al., (1983) due to the perceived inconsistencies of L_3 migrating out of the agar blocks. The simplified test contained a shorter anthelmintic incubation period using exsheathed L_3 for 3 h at room temperature, before transferring to migration chambers fitted with 20 µm nylon mesh in a 48-well plate. The larvae were left to migrate for 16 h at room temperature and the percentage migration at a particular concentration compared to those in a control well containing no anthelmintic (Figure 25). Tests using *T. colubriformis* in combination with IVM indicated 8 - 80% migration inhibition across concentrations ranging from 0.1 – 100 µg ml^{-1} IVM.

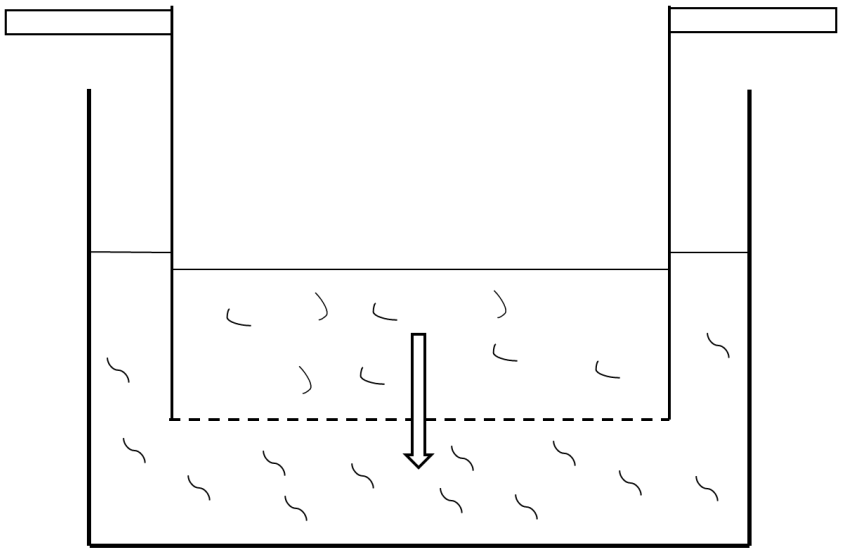

Figure 25: Diagram of migration apparatus as adapted from Wagland et al (1992). Larvae paralysed by the test substance are unable to migrate through the mesh and so are retained in the migration chamber. Larvae not paralysed by the test substance are able to migrate down through the mesh into the plate well below, in the direction indicated by the arrow.

Rabel et al., (1994) also conducted a series of comparisons to investigate operational factors such as the optimum number of larvae per well, dispensing error, effect of

sieve blockage and the effect of solvent (DMSO; dimethyl sulfoxide) used to dissolve test substances. The results generated were highly reproducible and showed operational advantages over previous studies (Rabel et al., 1994). The migration method originally developed by Wagland et al., (1992) (as modified by Rabel et al., 1994) has subsequently been used to investigate the effect of condensed tannins on *T. colubriformis* (Molan et al., 2000) and that of LEV on *Oesophagostomum denatatum* (Martin et al., 2003). It has also been used successfully in conjunction with abomasal mucus to ascertain the immunomodulatory effect of mucus from previously-infected calves on migration of *O. ostertagi* L_3 (Claerebout et al., 1999).

Recently, a further adapted method was published by Demeler et al., (2010b) designed to test the effect of ML anthelmintics on the migration of *O. ostertagi* and *C. oncophora* L_3. This study focused on optimisation of three tests; namely, the larval migration inhibition test (LMIT), the larval development test (LDT) and, for use with adult worms, a motility meter test. The LDT was considered to be a rather fragile system, requiring consideration of a number of confounding factors, and the use of fresh, anaerobically stored faeces, whilst the motility meter was considered not be sufficiently sensitive for use with larvae. However, the LMIT results showed considerable promise as a potential diagnostic test, with scope for further development. The LMIT method described by Demeler et al., (2010b) requires an initial incubation period of 24 h, followed by a migration period of 24 h utilising a mesh size (20 - 28 µm) that is dependent on the nematode species being examined: 28 µm was selected for the test involving cattle nematode species. A five-fold difference in LD50 estimates was observed between IVM-susceptible and IVM-

resistant *C. oncophora* isolates, although an IVM-resistant *O. ostertagi* isolate was not available for comparison against an IVM susceptible isolate. This LMIT method was then subjected to a 'ring test' across a number of laboratories; however, 'definitive' LD50 thresholds for determining IVM resistance status of a particular monospecific isolate remained to be established (Demeler et al., 2010a). This was due, in part, to the eight-fold difference in LD50 estimates observed during the 'ring test' using the same IVM-resistant and susceptible *C. oncophora* isolates that showed a five-fold difference in the original study (Demeler et al., 2010a; Demeler et al., 2010b).

This chapter describes optimisation experiments of the LMIT, based primarily on the principles established by Wagland et al. (1992), for the purpose of screening ML sensitivity of field populations of cattle nematodes. As outlined above, various protocols have been developed for larval migration and paralysis tests for use with single nematode species, with the caveat that further investigation was required for mixed-species isolates, a likely consideration when assessing field samples. As basic laboratory conditions for the test varied amongst published protocols (for example, the use of sheathed versus exsheathed L_3, mesh pore diameter and anthelmintic solvent), the impact of these parameters were examined in detail here. Single species nematode isolates (comprising *O. ostertagi* and *C. oncophora* L_3), categorised as IVM resistant based on FECRT analysis and laboratory isolates never exposed to ML anthelmintics (Coop et al., 1979; Van Zeveren et al., 2007a), were used to establish the value of the LMIT in differentiating L_3 of varying ML sensitivities. In addition, L_3 derived from two mixed-species isolates (FI001 and FI004), previously

characterised as IVM resistant by FECRT and CET (McArthur et al. 2011; Bartley et al., 2012), were examined to inform on the utility of the LMIT in the characterisation of IVM sensitivity of mixed-species field samples.

5.2 Materials and Methods

5.2.1 Parasitic isolates

Six nematode isolates were used to evaluate the LMIT for use with cattle nematode larvae. Details of the provenance and sensitivity of these isolates is summarised in Table 29. Two test populations comprised mixed-species field isolates, containing both *O. ostertagi* and *C. oncophora* spp. The remaining four isolates comprised single nematode species; two isolates monospecific for *O. ostertagi* and the two isolates monospecific for *C. oncophora*. The two field isolates (FI001 and FI004) were isolated from two Scottish cattle farms in 2010 (located in Dumfriesshire and Ayrshire, respectively). When first assessed, FI001 comprised 15% *O. ostertagi*, 85% *C. oncophora*, whereas FI004 comprised 38% *O. ostertagi* and 62% *C. oncophora*. In a FECRT using subcutaneously administered IVM (0.2 mg kg^{-1} body weight, BW), the isolates exhibited FEC reductions at 14 days post administration of 72.4% and 87.3%, respectively (McArthur et al., 2011). Both isolates were subjected to a controlled efficacy test (described in Chapter 4; (Bartley et al., 2012)) where IVM was administered as a topical pour-on formulation and subcutaneously, and moxidectin (MOX) was administered as a pour-on preparation. Based on enumeration of adult worm burdens, percentage efficacies for FI001 were 38%, 64% and 31% and for FI004 were 10%, 0% and 74%, for injectable IVM, pour-on IVM and pour-on MOX, respectively. As a result, the presence of IVM and MOX resistant *C. oncophora* in both isolates was confirmed. In the CET, *O. ostertagi* worms present in both isolates were confirmed as being ML-sensitive with the percentage mean reduction in adult worm burden greater than 99.5%.

O. ostertagi isolates were kindly provided by Professors Edwin Claerebout and Peter Geldhof (Ghent University, Belgium): one isolate was previously shown to sensitive to IVM *in vivo* (Van Zeveren et al., 2007a) and the other isolate resistant to IVM treatment *in vivo* (Van Zeveren et al., 2007a). Examination of larvae upon arrival at Moredun Research Institute revealed that both isolates contained 4% *C. oncophora* larvae and 96% *O. ostertagi* larvae. The proportion of each species remained unchanged following passage through helminth-naïve calves as outlined below. The IVM-sensitive isolate (Oo IVM-S) was originally isolated from a Belgian dairy farm in 1987 and stored in liquid nitrogen for the next six years. Since 1993, the isolate had been maintained via passage of helminth-naïve calves (Van Zeveren et al., 2007a). The isolate was confirmed to be sensitive to IVM treatment following a FECRT in which IVM was administered subcutaneously at the manufacturer's recommended dose rate (0.2 mg kg^{-1} body weight, BW). No eggs were observed by FEC analysis conducted at seven and 14 days post treatment, confirming IVM sensitivity. Calves tested in this FECRT were necropsied three days later (i.e. 17 days after IVM treatment) and adult *O.ostertagi* worms were not recovered from the abomasum (Van Zeveren et al., 2007a). The IVM-resistant *O. ostertagi* isolate (Oo IVM-R) was generated through repeated exposure of the IVM-sensitive isolate (Oo IVM-S) to increasing levels of IVM *in vivo* over ten successive generations (Van Zeveren et al., 2007a). Following anthelmintic administration, eggs passed out in faeces were collected and cultured to L_3. These larvae were then used to infect more calves for the next round of selection. For seven generations, the dose rate of IVM administered increased across sub-therapeutic levels of IVM, from 0.0125 mg kg^{-1} BW, to 0.1 mg kg^{1} BW. For the final three generations, infected calves were treated

at the manufacturer's recommended dose rate (0.2 mg kg^{-1} BW). After a total of ten generations, a FECRT was conducted to examine IVM sensitivity of the isolate. Based on FEC analysis at 14 days after IVM treatment, a percentage efficacy of 65.4% (range 0 – 94%) was identified (Van Zeveren et al., 2007a). A later FECRT and CET showed FEC reduction of 68% at 14 days after IVM administration and the percentage mean reduction in adult worm burden compared to untreated control animals was 84%, confirming resistance (de Graef et al., 2012).

The IVM sensitive *C. oncophora* isolate (Co IVM-S) is a Moredun Research Institute laboratory isolate that was isolated locally prior to 1979 (thus never exposed to ML treatment). This isolate had been stored in liquid nitrogen and annually passaged through helminth-naïve lambs and calves (Coop et al., 1979). To provide a monospecific IVM-R *C. oncophora* isolate (Co IVM-R), 25,000 larvae from F.I.001 isolate (15% *O. ostertagi*, 85% *C. oncophora*) were administered *per os* to a 4 month-old helminth-naïve, Greyface male lamb. Only the *C. oncophora* larvae were able to produce a patent infection, as previously reported by Keith (1953), and this was confirmed via morphological identification of larvae following collection from coproculture.

Table 29: Provenance and IVM sensitivity status of six nematode isolates used in LMIT analysis. Isolates comprised two O. ostertagi isolates (kindly provided by University of Ghent), two C. oncophora isolates and two mixed-species, field isolates. A monospecific IVM-R C. oncophora isolate was generated by passaging field isolate FI001 L3 through a helminth-naïve lamb. IVM sensitivity of each isolate was determined based on published efficacy data (with the exception of Co IVM-S). In vivo efficacy data is based on reductions in adult worm burden following subcutaneous administration of IVM (0.2 mg kg-1 BW), compared to untreated control animals. IVM efficacy against O .ostertagi in the two field isolates was demonstrated to be >99.5%.

Isolate code	Species present	Provenance of isolate	*In vivo* efficacy of IVM	References
Oo IVM-R	96% *O. ostertagi* 4% *C. oncophora*	Experimentally selected over 10 successive generations to induce IVM-R, maintained since 2007	IVM-Resistant; 84% reduction in adult burden after IVM treatment	(Van Zeveren et al., 2007a)
Oo IVM-S	96% *O. ostertagi* 4% *C. oncophora*	Belgian dairy farm, isolated in 1987	IVM- Sensitive; 100% reduction in adult burden after IVM treatment	(Van Zeveren et al., 2007a)
Co IVM-S	100% *C. oncophora*	Moredun laboratory isolate, isolated prior to 1979	IVM-Sensitive; never exposed to ML anthelmintics	(Coop et al., 1979)
Co IVM-R	100% *C. oncophora*	Monospecific isolate generated via passage of FI001, passaged in 2011	IVM-Resistant; 38% reduction in adult burden after IVM treatment	(Bartley et al., 2012)
FI001	15% *O. ostertagi* 85% *C. oncophora*	Farm 001, Dumfries, Scotland; isolated in 2010	IVM-Resistant *C. oncophora;* 38% reduction in adult burden after IVM treatment	(Bartley et al., 2012)
FI004	38% *O. ostertagi* 62% *C. oncophora*	Farm 004, Ayrshire, Scotland; isolated in 2010	IVM-Resistant *C. oncophora*; 10% reduction in adult burden after IVM treatment	(Bartley et al., 2012)

5.2.2. Larval collection and maintenance

O. ostertagi and mixed-field isolates (Oo IVM-S and IVM-R, FI001, FI004) were passaged through helminth-naïve male Holstein Friesian calves, aged 3-4 months.

Calves were administered with 50,000 L_3 *per os* and FEC (Jackson, 1974) examined weekly to monitor infection. Once infection was observed to be patent (usually 21 days post infection, verified by the appearance of eggs in FEC analysis), calves were harnessed to collect faeces and collection bags changed twice daily. Coprocultures of collected faeces were conducted as follows: faeces were mixed with an equal volume of vermiculite, formed into balls, approximately 5 cm in diameter, placed into plastic trays, covered with a perforated polythene bag to allow air circulation and incubated at 15 °C for 14 - 17 days.

Cooperia isolates (Co IVM-S and IVM-R) were passaged through helminth-naïve male Greyface lambs, aged approximately 4 months. The lambs were infected with 25,000 L_3 *per os*, and following confirmation of patent infection by FEC analysis (21 days post-infection) were harnessed to collect faecal output and collection bags changed daily. After collection, faecal pellets were transferred to polythene lined trays to a maximum depth of 5 cm, covered with a perforated polythene bag and incubated for 14 days at 15 °C.

Following coproculture, all larvae were extracted using Baermann apparatus as described in Chapter 3 (M.A.F.F., 1986). Larvae were enumerated by removal of a 1 ml aliquot, re-suspended with 9 ml tap water in a volumetric flask, from which ten to 20 aliquots of 10 µl were taken and counted under x 100 magnification. L_3 were identified to species using morphological keys (M.A.F.F., 1986) and also with reference to Hansen and Shivnani (Hansen and Shivnani, 1956). The presence of non-strongyle or rhabditoid free-living larvae was noted on each flask and was recorded as below 1% in all batches. The number of L_3 per ml was calculated for

each batch and the density of L_3 per flask adjusted to a maximum of 4,000 L_3 ml^{-1}. Flasks were stored at 4 °C and examined regularly for fungal growth and the water replenished weekly. In all cases, L_3 were assessed in the LMIT within 3 months of extraction from faeces. Unless otherwise stated, L_3 were adjusted to a final concentration of 1,000 L_3 ml^{-1} prior to incubation with IVM.

5.2.3 Larval exsheathment protocol

L_3 were exsheathed in sodium hypochlorite solution immediately prior to use, using the following procedure. After enumeration to determine the volume of required, an aliquot was removed from the L_3 culture flask into a 15 ml pointed-end centrifuge tube (Fisher Scientific Ltd) and the volume adjusted using ultrapure water to 10 ml. To this, 800 µl of sodium hypochlorite solution (0.5% w/w, Milton™, Ceuta Healthcare Ltd) were added, gently inverted three times and incubated for 3.5 min at room temperature. Exsheathed L_3 were centrifuged for 2 min at 203 x *g*, then washed and centrifuged three times in ultrapure water (H_2O) to remove excess sodium hypochlorite solution. Successful exsheathment was confirmed by observation of 10 µl of L_3 solution under x 40 magnification.

5.2.4. Baermannisation protocol

Two millilitres ultrapure H_2O were added to one well of a 6-well cell culture plate (Corning). Sheathed or exsheathed L_3 were pipetted into a moistened Baermann apparatus (Baermann, 1917) and the entire apparatus transferred to the 6-well plate. The L_3 were incubated for 2 h at 22 °C before the device was removed from the plate

and the migrated L_3 recovered. The L_3 were enumerated as before and concentration adjusted to 1,000 L_3 ml^{-1}.

5.2.5 LMIT optimisation

5.2.5.1 Determination of optimum mesh pore size

The selection of mesh pore size needs to be considered carefully to avoid confounding test results as too small a pore size will result in a failure of viable L_3 to migrate and too large a pore size may allow dead or paralysed L_3 to simply fall through the mesh leading to false positive results. Initially, filter equipment was kindly provided by Dr. Janina Demeler (Freie Universitat, Berlin). Six Plexiglass tubes (Evonik Industries), each measuring 20 mm in length and 12 mm in diameter, were fitted with nylon mesh of either 25 µm or 28 µm pore diameter (HPC Gears Ltd) to form migration chambers and attached to plastic sticks with water-resistant Superglue™ (Loctite). Figure 26 shows two of the completed migration sticks.

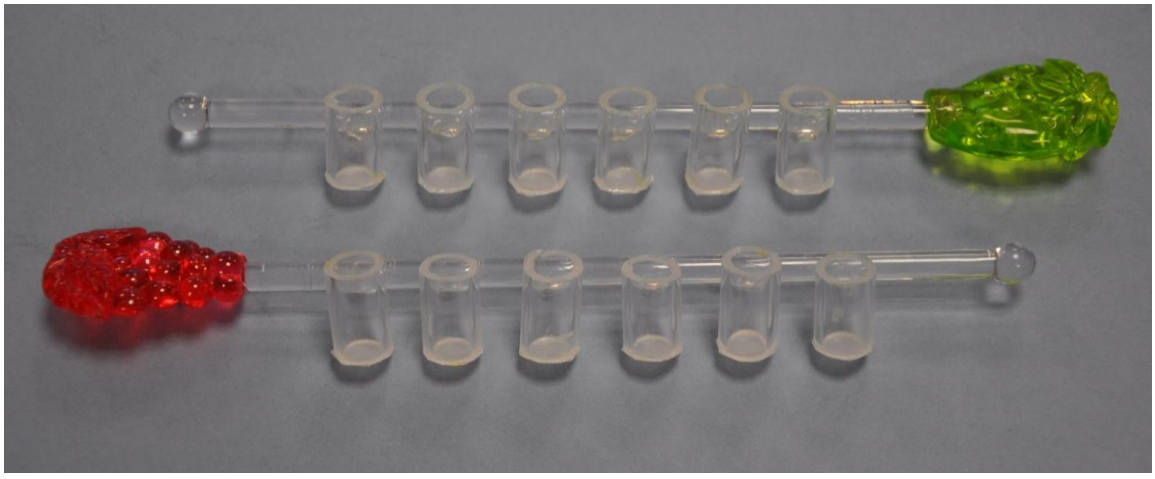

Figure 26: Two complete migration sticks, each consisting of six Plexiglass chambers fitted with 25 µm mesh.

Prior to each use, sticks were examined under a microscope at x 40 magnification for any holes or yellowing of the glue binding to the mesh, which may indicate perishing. Any sticks found to have chambers containing holes were removed from use, the mesh replaced with fresh material, fixed with Superglue ™ and left to dry for 24 h before being used. As IVM has previously been shown to bind to glass and plasticware (Tway et al., 1981), for use in the optimised assays, sticks were colour coded so migration chambers used for negative control wells (H_2O or DMSO only wells) were never exposed to IVM. Findings by Demeler et al., (2010a) suggested that *O. ostertagi* L_3 did not migrate as well in negative control wells as *C. oncophora* L_3. For this reason, *O. ostertagi* L_3 were used for the majority of the optimisation tests, to ensure sufficient levels of migration were achieved in control wells.

To select the most appropriate mesh pore size for use with *O. ostertagi* and *C. oncophora* L_3, a heat-treatment experiment was conducted, using heat-treated L_3 as a negative control. An aliquot of 1,200 *O. ostertagi* (Oo IVM-R) L_3 were removed from culture, exsheathed and adjusted to 1,000 L_3 ml^{-1} with ultrapure H_2O in a total volume of 1,200 µl. From this, 800 µl L_3 were pipetted into an Eppendorf, lidded and sealed with Parafilm® (Pechiney Plastic Packaging Company) and incubated in a pre-heated water bath at 70 °C for 20 min. The remaining 400 µl was sealed in an Eppendorf with Parafilm® and incubated at room temperature for 20 min. After this, 2 ml ultrapure H_2O were added to each of 6 wells in the first and third rows (rows A and C) of a 24-well cell culture plate (Corning), as shown in Figure 27. To row A, a

migration stick consisting of 6 migration wells fitted with 25 µm nylon mesh was added, with a similar stick fitted with 28 µm mesh added to row C. Next, 90 µl aliquots of heat killed or live L_3 were taken from the appropriate Eppendorf and gently pipetted to the inside of an appropriate migration chamber. The plates were then covered with foil and incubated in the dark for 2 h at 26 °C.

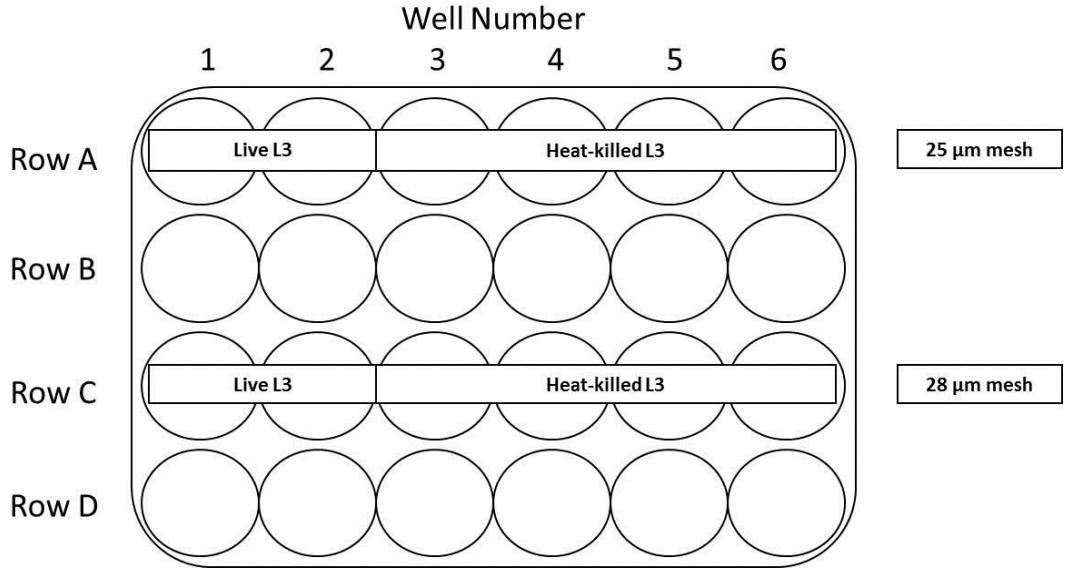

Figure 27: Diagram of plate layout for heat-treatment experiment. Migration sticks were placed in rows A and C. Live L3 were added to wells A1, A2, C1 and C2. Heat-treated L3 were added to wells A3 – 6 and wells C3 – 6. Following incubation, migration sticks were lifted, inverted over rows B and D and non-migrated larvae washed into the respective wells.

Following incubation, the plates were removed and uncovered. Starting with the stick in row C, the outside of each chamber was gently washed with 600 µl ultrapure H_2O to wash any L_3 that had potentially migrated, but adhered to the outside of the chamber, into the appropriate well. The stick was then inverted over Row D and non-migrated L_3 washed into the corresponding well with 2 ml ultrapure water. This was then repeated with the stick in Row A, so every well containing migrated L_3 (for example, A1) had a corresponding well containing non-migrated L_3 (for example,

B1). After washing, the mesh was checked under a microscope to ensure no L_3 were adhering to the mesh. The plates were transferred to an inverted stereomicroscope and L_3 examined under x 100 magnification to ensure all heat-treated L_3 were dead and live L_3 were motile. Once this had been established, and to aid counting, 200 µl molecular grade ethanol (EtOH; Sigma Aldrich) were added to each well containing live L_3 and the numbers of migrated and non-migrated L_3 enumerated. The same protocol was repeated using sheathed L_3 and the comparison repeated on two separate occasions.

5.2.5.2. Determination of optimum DMSO concentration

Due to the insolubility of IVM in water, a solvent, such as DMSO, must be used to sufficiently dissolve IVM in solution, and without adversely affecting the L_3 being tested in the LMIT. To ascertain the effect of DMSO concentration on L_3 in the absence of IVM, the following experiment was conducted. Approximately 1,400 *O. ostertagi* (IVM-S) L_3 were removed from culture and re-suspended in ultrapure H_2O in a total volume of 1,400 µl. To dark amber Eppendorfs (Axygen), 2, 4, 6 and 8 µl DMSO (Sigma Aldrich) were added to 8, 6, 4, 2 µl ultrapure water, respectively, followed by 190 µl L_3 culture, to produce a range of DMSO concentrations of 1, 2, 3 and 4%. For 5% DMSO concentration, 10 µl DMSO were added to 190 µl L_3 solution, and 10 µl ultrapure water added to an Eppendorf containing 190 µl L_3, as a negative control. All Eppendorfs were vortexed briefly to ensure mixing and incubated in the dark at 26 °C for 2 h. To five darkened 30 ml universal containers (Sterilin), 40, 80, 120, 160 and 200 µl DMSO were added, followed by 3.96, 3.92, 3.84 and 3.8 ml ultrapure water, respectively. All universals were then vortexed

briefly to ensure mixing. For two wells on a 24-well plate, 1,910 µl of a particular DMSO dilution were added, with two additional wells filled with 1,910 µl ultrapure water to serve as negative controls. A migration stick, comprising six migration chambers fitted with 25 µm mesh was added to rows A and C as for the heat-treatment experiment. After brief mixing by pipette, two 90 µl aliquots of the incubated L_3 and IVM solution were added gently down the inside of two migration chambers. The plates were covered in tin foil and incubated for a 2 h at 26 °C. Next, the plates were removed from the incubator and the non-migrated L_3 washed into the corresponding well as described in Section 5.2.5.1. The contents of each well were fixed with 200 µl 100% molecular grade ethanol. All L_3 in migrated and non-migrated wells were counted under x 100 magnification on an inverted stereomicroscope.

5.2.6. Range of ivermectin solutions tested in LMIT

Table 30 summarises the range of IVM concentrations evaluated in this study. All solutions were kept at room temperature in darkened containers to prevent ML degradation (Halley et al., 1993). Under these conditions, the stock solution (3000 µg ml^{-1} IVM) was kept for a maximum of 2 months before replacement, with fresh dilutions of the remaining concentrations made weekly. Moreover, only dark amber Eppendorf tubes were used and plates covered in aluminium foil whilst the tests were conducted. In addition to the IVM concentrations shown, L_3 were also incubated in 3% DMSO only and ultrapure water only as negative controls. Molarity was calculated using a combination of molecular weights of ivermectin B1a and B1b (474 g and 466 g, respectively) as the data sheet supplied by Sigma Aldrich

displayed a composition of 91% B1a and 3% B1b. Using these values, the molarity of the stock solution was calculated to be 6.73×10^{-3} M.

Table 30: IVM concentrations used in LMIT, expressed as µg ml-1 and as molarity (M)

Working concentration IVM (µg ml^{-1})	Final concentration IVM (µg ml^{-1})	Multiplication Factor	Molarity (M)Working Concentration	Final molarity (M) in well
3000	150	-	6.73×10^{-3}	3.37×10^{-4}
1500	75	0.5	3.37×10^{-3}	1.68×10^{-4}
600	30	0.4	1.35×10^{-3}	6.73×10^{-5}
300	15	0.5	6.73×10^{-4}	3.37×10^{-5}
60	3	0.2	1.35×10^{-4}	6.73×10^{-6}
30	1.5	0.5	6.73×10^{-5}	3.37×10^{-6}
20	1	0.67	4.49×10^{-5}	2.24×10^{-6}
10	0.5	0.5	2.24×10^{-5}	1.12×10^{-6}
5	0.25	0.5	1.12×10^{-5}	5.61×10^{-7}
1	0.05	0.2	2.24×10^{-6}	1.12×10^{-7}

5.2.7. Optimised LMIT protocol

After analysis of the above optimisation steps, the finalised procedure was as follows: approximately 4,800 L_3 were removed from culture and re-suspended in ultrapure water in a total volume of 2,800 µl. To a dark amber Eppendorf tube containing 6 µl IVM+DMSO dilution, 194 µl L_3 solution were added, mixing by

pipette each time to ensure an even distribution of L_3 as possible. In addition, L_3 were added to Eppendorf tubes containing 6 µl DMSO only (no IVM present, solvent control) and Eppendorf tubes containing 6 µl ultrapure water only (no IVM or DMSO control). Final IVM concentrations used were 0.05, 0.25, 0.5, 1, 1.5, 3, 15, 30, 75, 150 µg ml^{-1} IVM. All Eppendorf tubes were vortexed briefly to ensure mixing and incubated in the dark for 2 h at 26 °C. Prior to completion of the incubation, reagents for the migration plates were prepared by adding 300 µl IVM + DMSO dilution to 9,700 µl ultrapure water in a darkened universal and vortexed briefly. To conduct the test in duplicate, 1,910 µl of IVM + DMSO dilution were added to two wells on a 24-well cell culture plate, repeated for each IVM concentration, along with two 3% DMSO only wells, and two ultrapure water only wells (Figure 28). A migration stick, comprising six migration chambers fitted with 25 µm mesh was immersed in the solution of rows A and C. After brief mixing by pipette, two 90 µl aliquots of the incubated L_3+IVM solution were gently pipetted down the inside of two migration chambers. The plates were covered in aluminium foil and incubated for 2 h at 26 °C.

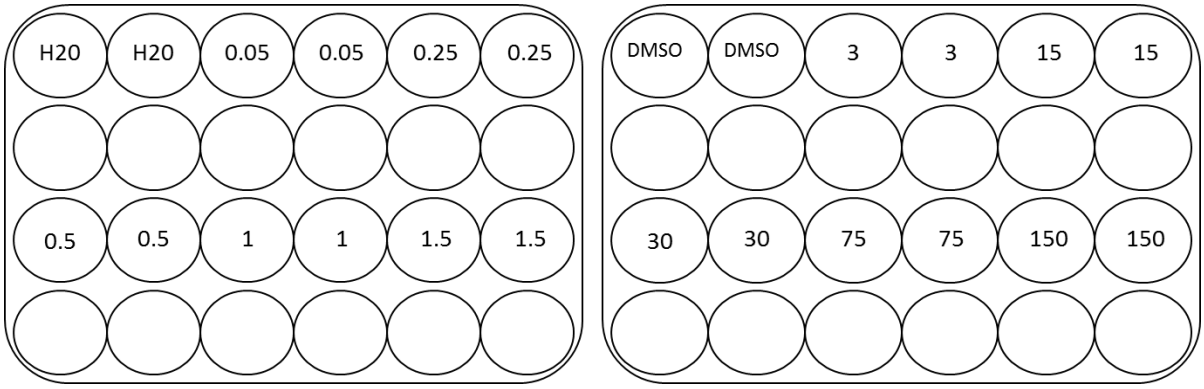

Figure 28: Layout of LMIT concentrations (in µg ml-1 IVM) on a 24-well plate. All IVM concentrations were conducted in duplicate and a set of control wells (either ultrapure water or 3% DMSO) were included on each plate.

After 2 h, the plates were removed from the incubator and uncovered. The sticks were removed, L_3 washed, fixed and counted as described in Section 5.2.5.1. The susceptible *C. oncophora* isolate (Co IVM-S) was examined on 11 separate occasions in duplicate and both *O. ostertagi* isolates were examined 10 times in duplicate (Oo IVM-S and IVM-R). FI004 was examined eight times in duplicate and the resistant *C. oncophora* isolate (Co IVM-R1) and FI001 were examined five times in duplicate. This protocol was also conducted using both sheathed and exsheathed *O. ostertagi* L_3 (Oo IVM-S and IVM-R) on two occasions in duplicate and with Baermannised and non-Baermannised L_3 (Oo IVM-S and IVM-R). Due to a previous published study indicating that differences in levels of *O. ostertagi* migration could be observed after a storage period of two months (Geerts et al., 1989), Oo IVM-S was tested using L_3 which had been stored for one week at 4^oC and L_3 which had been stored for two months at 4^oC. This comparison was also conducted using freshly extracted *C. oncophora* L_3 (stored for one week at 4 °C) and

L_3 stored for 6 months at 4 °C (Co IVM-S). Both comparisons were conducted twice, in duplicate.

5.2.8. Data analysis

Percentage migration was calculated by ((T-(M/T) x 100, where T equals "Total number of L_3 per well" (the number of L_3 migrated plus number of L_3 that failed to migrate) and M represents "Number of migrated L_3 per well". A minimum threshold of 85% migration in both DMSO- and water-only control wells was established for inclusion in further data analysis and on this basis, no results were excluded. This threshold had been previously used by Demeler et al (2010b) to account for the potentially lower migration of *O. ostertagi* L_3 in comparison to *C. oncophora* L_3 and was adopted for this study to aid comparison of results between studies. Dose response curves were generated by averaging percentage migration across both wells at each concentration. Two sample Kolmogorov-Smirnov tests (Chakravarti et al., 1967) were conducted where appropriate to test for statistical differences in distributions between isolates. LD50 estimates were generated using probit analysis in R statistical environment (version 3.0.1), using packages 'MASS' (version 7.3-26) and 'epicalc' (version 2.15.1.0). Graphs were drawn with Microsoft Excel 2007 and with R using package 'ggplot2' (version 0.9.3.1).

5.3 Results

5.3.1. Determination of optimum mesh pore size

Data derived from the experiments designed to determine optimum mesh pore size for use with *O. ostertagi* (Oo IVM-S) larvae are shown in Figure 29. All L_3 in the negative control wells (i.e. live L_3 not killed by prior heat treatment) were observed to be motile. The mean number of L_3 per set of test wells was 125 L_3 (± 6 standard error of mean; SEM). L_3 migration in the negative control wells ranged between 91.8 and 97%. With heat-treated L_3, the percentage of L_3 falling through the mesh was lowest when sheathed larvae were combined with a 25 µm pore mesh (mean 1% ± 0.3 SEM), compared to the highest level of "fall through" (mean 5.1% ± 1.4 SEM) observed with exsheathed L_3 tested with the 28 µm pore mesh. Differences observed between groups of sheathed and exsheathed larvae exposed to different mesh sizes were not statistically significant ($p > 0.05$). On the basis of these results, a 25µm mesh was selected for use in the optimised tests.

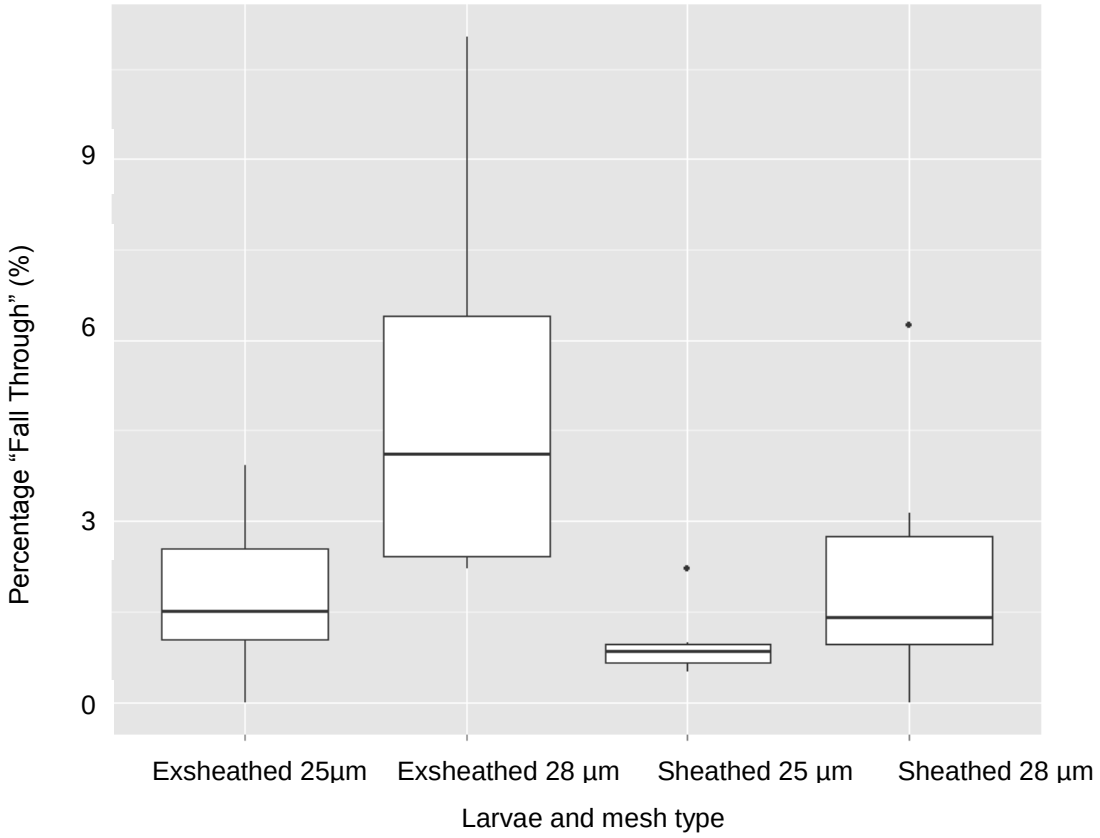

Figure 29: Box plot displaying the percentage 'fall through' of heat-treated sheathed and exsheathed *O. ostertagi* L_3 exposed to mesh sizes of 25µm and 28 µm. Lines in the middle of the boxes indicate median percentage 'fall through' for each set of L_3, with whiskers indicating maximum and minimum values for each group (n = 8).

5.3.2 Determination of optimum DMSO concentration

Data from the experiment performed to determine optimum DMSO concentration for use in the LMIT are shown in Figure 30. Sheathed and exsheathed Oo IVM-S L_3 demonstrated high levels of migration at all concentrations of DMSO tested (1 - 5% v/v %), with mean percentage migrations of 97.8% (± 1.3 SEM) for sheathed L_3 and 96.6% (± 1.4 SEM) for exsheathed L_3. Percentage migration with sheathed and exsheathed L_3 ranged from 89.5 - 100%. The lowest percentage migration (89.5%) was observed at 4% and 5% DMSO with exsheathed and sheathed L_3, respectively.

Sheathed L_3 samples migrated at levels of up to 100% at concentrations ranging from 1 - 4% DMSO, whereas 100% migration was not observed with exsheathed L_3 beyond a concentration of 3% DMSO. Based on these results, a DMSO concentration of 3% was selected for use in the optimised LMITs.

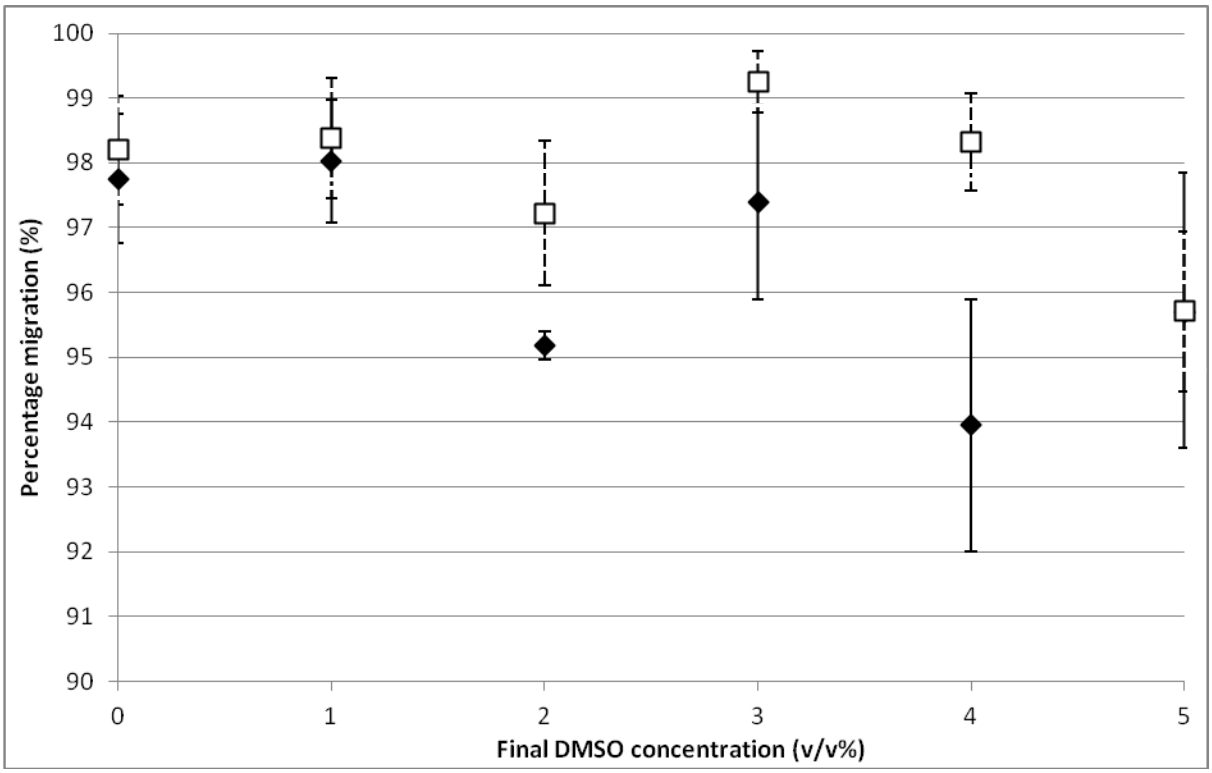

Figure 30: Mean percentage migration of *O. ostertagi* L_3 incubated in increasing concentrations of DMSO, conducted on two occasions, in duplicate. Open and closed symbols represent sheathed L_3 and exsheathed L_3, respectively. Error bars represent standard error of mean (SEM) for percentage migration at each concentration (n=4), dashed lines for sheathed L_3 and solid lines for exsheathed L_3.

5.3.3. Comparison of sheathed and exsheathed L_3, with or without a prior Baermannisation step

An evaluation was made of the impact of IVM+DMSO on sheathed and exsheathed L_3 from *O. ostertagi* isolates Oo IVM-R and Oo IVM-S, in addition to the impact of including a pre-LMIT Baermannisation step. The mean number of L_3 counted per set

of wells (i.e. migrated and non-migrated) was 137 (± 3 SEM) across all samples. LD50 estimates generated from this comparison are displayed in Figure 31.

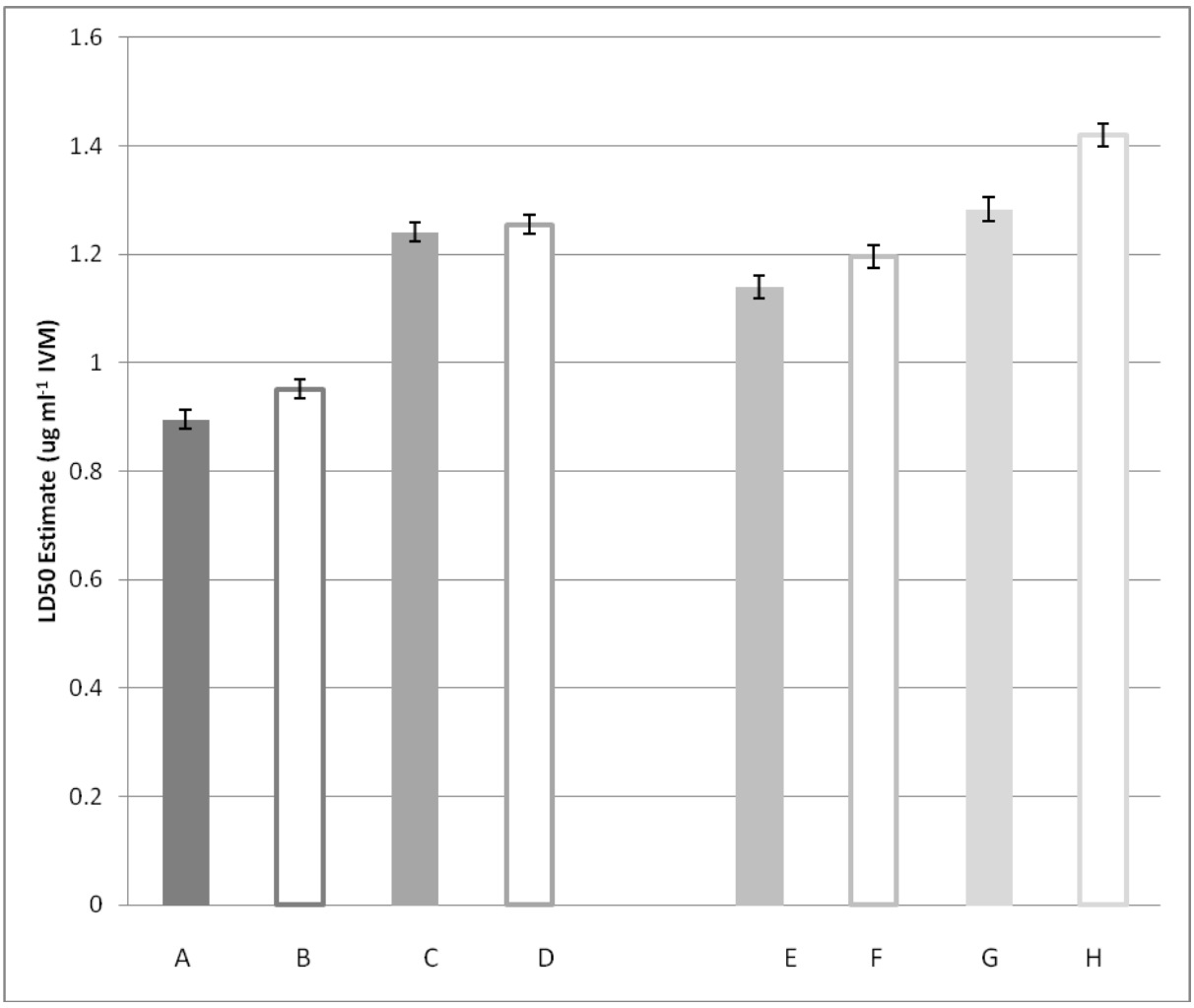

Figure 31: LD50 estimates from optimisation analysis. Bars A – D represent L3 from Oo IVM-S, and bars E – H represent L3 from Oo IVM-R. Bars A, B and E, F indicate estimates from sheathed L3, and bars C, D and G, H indicate estimates from exsheathed L3. Filled bars represent samples in which L3 were Baermannised prior to use, open bars represent L3 not Baermannised prior to use. Error bars indicate the standard error of the LD50 estimates.

The highest LD50 estimates were achieved using exsheathed L_3 which were not Baermannised prior to use (3.5 and 4.5 µg ml^{-1} IVM; Oo IVM-S and Oo IVM-R,

respectively). The lowest LD50 estimates were found with Baermannised, sheathed L_3 (1.1 and 3.3 µg ml^{-1} IVM; Oo IVM-S and Oo IVM-R, respectively). Migration in the H_2O-only control wells ranged from 87 to 99%, with a mean migration of 94.4% (± 1% SEM) and 93.2% (± 0.6% SEM) for Oo IVM-S and Oo IVM-R, respectively. Migration was found to be higher in the DMSO-only control wells, ranging from 93 to 99% with a mean migration of 97.14% (± 0.2% SEM) and 96.4% (± 0.4% SEM) for Oo IVM-S and Oo IVM-R, respectively. In larval cultures where there are high proportions of dead L_3 or free-living larvae, a Baermannisation step may prove helpful, but given the high levels of migration observed without a preceding Baermannisation step here, and the low levels of free living larvae present in cultures (<1%), it was decided not to include this step in the optimised test. In addition, L_3 stimulated by the exsheathment process may be more likely to pass through the mesh due to the increase in activity compared to sheathed L_3. As a result, the effect of L_3 exsheathment may confound the LMIT results, as any observed migration cannot be wholly ascribed as being in response to IVM incubation. Consequently, the decision was made to use sheathed L_3 in the optimised test to avoid confounding.

5.3.4. Effect of age on migration of *Ostertagia ostertagi* and *Cooperia oncophora* larvae

The effect of prior length of time of storage of L_3 on migration was tested using Oo IVM-S and Co IVM-S (Figure 32). The mean number of L_3 counted across each set of wells was 146 (± 3 SEM). For Oo IVM-S L_3 stored for one week, mean percentage migration in the H_2O-only control wells was 97.7%, compared to 95.5% migration observed in DMSO-only control wells. With Oo IVM-S L_3 stored for two

months, mean percentage migrations of 97.4% and 96% were observed in H_2O-only and DMSO-only wells, respectively. LD50 estimates of 1.2 and 1.1 µg ml^{-1} IVM were generated for one week-old and two month-old L_3, respectively.

C. oncophora (Co IVM-S) L_3 stored for one week gave a mean percentage migration of 95.9% (± 0.96% SD) in H_2O-only wells and 97.3% (± 1.55% SD) migration in DMSO-only control wells. The mean number of L_3 counted across each set of wells was 217 L_3 (± 69). Larvae previously stored for six months at 4 °C showed a mean migration of 95% (± 0.82% SD) in H_2O-only wells and 93.8% (± 1.75% SD) mean migration in the DMSO-only wells. LD50 estimates were 3.2 and 3.3 µg ml^{-1} IVM for one week-old and six-month old L_3, respectively. For these studies, the age of L_3 was not considered a concern because all L_3 tested subsequently had been stored for less than three months following collection.

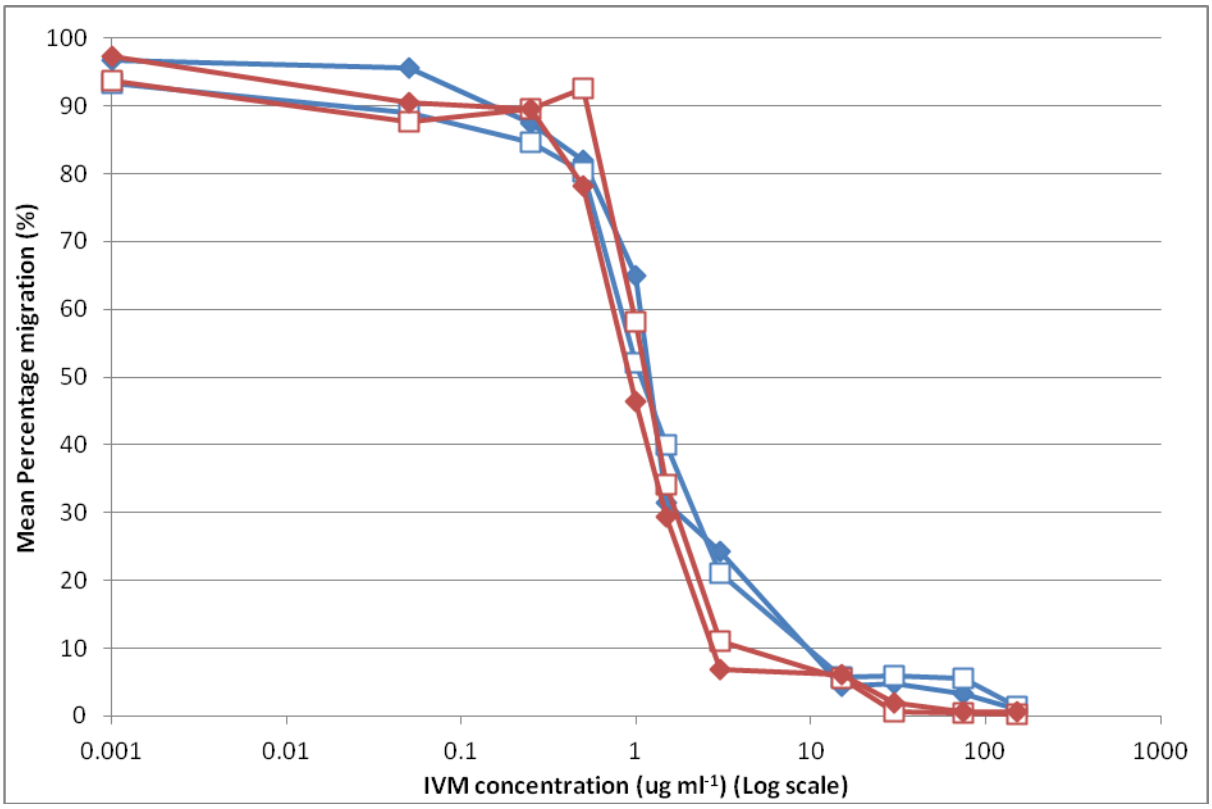

Figure 32: Dose response curves displaying the effect of storage on migration of *O.ostertagi* and *C. oncophora* L_3 across a range of IVM concentrations. Blue symbols represent Oo IVM-S L_3, with red symbols representing Co IVM-S L_3. Open symbols represent L_3 stored for one week at 4°C prior to LMIT, closed symbols indicate L_3 stored for two or six months prior to testing, for Oo IVM-S and Co IVM-S, respectively. Comparison completed on two occasions (four times in total).

5.3.5 Use of the optimised LMIT protocol to test parasite isolates

5.3.5.1 Optimised LMIT conducted with *Ostertagia ostertagi*

Dose response curves derived from the LMIT using the *O. ostertagi* isolates (Oo IVM-S and Oo IVM-R) are displayed in Figure 33. For Oo IVM-S, the mean number of L_3 per set of wells was 159 (± 40 SD), ranging from 81 to 337 L_3. With Oo IVM-R, the mean was 116 L_3 (± 22 SD). For both isolates, all control wells (i.e.

wells containing water only or DMSO only) showed migration in excess of the 85% threshold. For Oo IVM-S, the mean percentage migration for water-only control wells was 93.9%, with 95.2% mean migration achieved in wells containing DMSO only. In LMIT experiments with Oo IVM-R, mean percentage migrations of 94.2% and 95.3% were achieved in wells containing H_2O-only and DMSO-only, respectively.

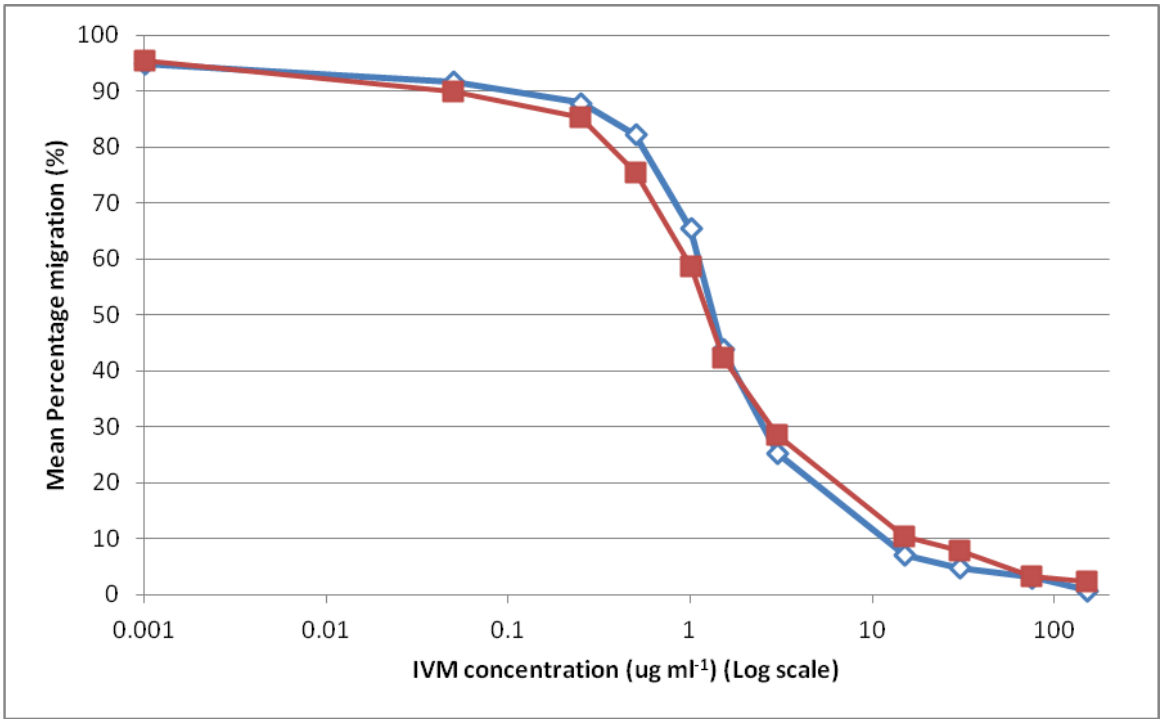

Figure 33: Mean percentage migration of two *O. ostertagi* isolates incubated in a range of IVM concentrations, conducted on ten occasions, in duplicate. Open and closed symbols represent IVM-sensitive isolate (Oo IVM-S) and IVM-resistant isolate (Oo IVM-R), respectively.

5.3.5.2 Optimised LMIT conducted with *Cooperia oncophora* isolates

The dose response curves derived from the LMIT using the two *C. oncophora* isolates (Co IVM-S and Co IVM-R) are displayed in Figure 34. With Co IVM-S, the mean number of L_3 per set of wells was 195 (± 64 SD) and was 193 (± 71 SD) with Co IVM-R. L_3 in all control wells for both isolates exhibited migration in excess of

the 85% threshold. With isolate Co IVM-S, mean percentage migrations of 92.8% and 91.8% were observed in wells containing water or DMSO, respectively. For Co IVM-R, a mean percentage migration of 93.9% was observed in wells containing water only and 92.8% migration observed in wells containing only DMSO.

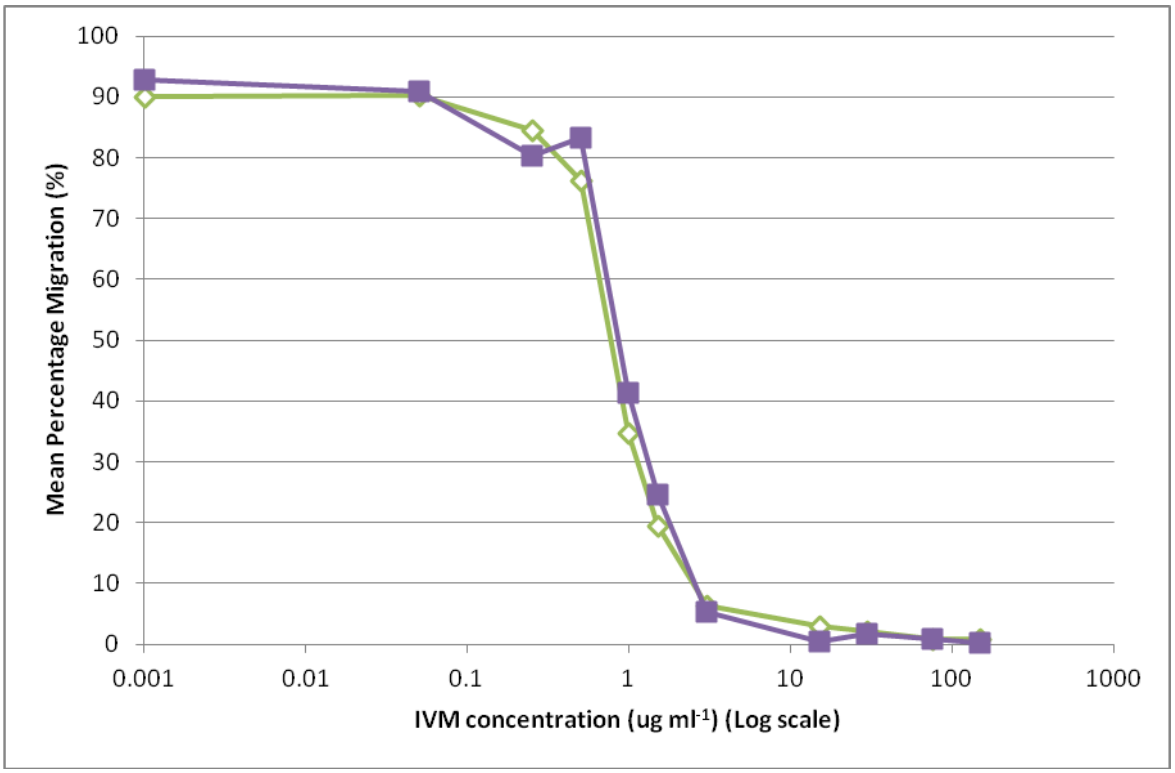

Figure 34: Mean percentage migration of two *C. oncophora* isolates incubated in a range of IVM concentrations. Open and closed symbols represent IVM-sensitive isolate (Co IVM-S) and IVM-resistant isolate (Co IVM-R), respectively. LMIT was conducted 11 times in duplicate with Co IVM-S (n=22) and five times in duplicate for Co.IVM-R (n=10)

5.3.5.3 Optimised LMIT conducted with two field isolates

The dose response curves derived using L_3 from the two mixed species field isolates (FI001 and FI004) are displayed in Figure 35. With this comparison, the number of L_3 per set of wells was 147 (± 38 SD) for FI004 and for to FI001, mean 108 (± 23 SD). Larval migration in all control wells for both isolates was in excess of the 85%

threshold set. For FI001, the mean percentage L_3 migration in the control wells was 92.6% and 88.9% for H_2O-only and DMSO-only wells, respectively. For FI004, mean percentage L_3 migration achieved in control wells was 91.3% and 93.6% for H_2O-only and DMSO-only wells, respectively.

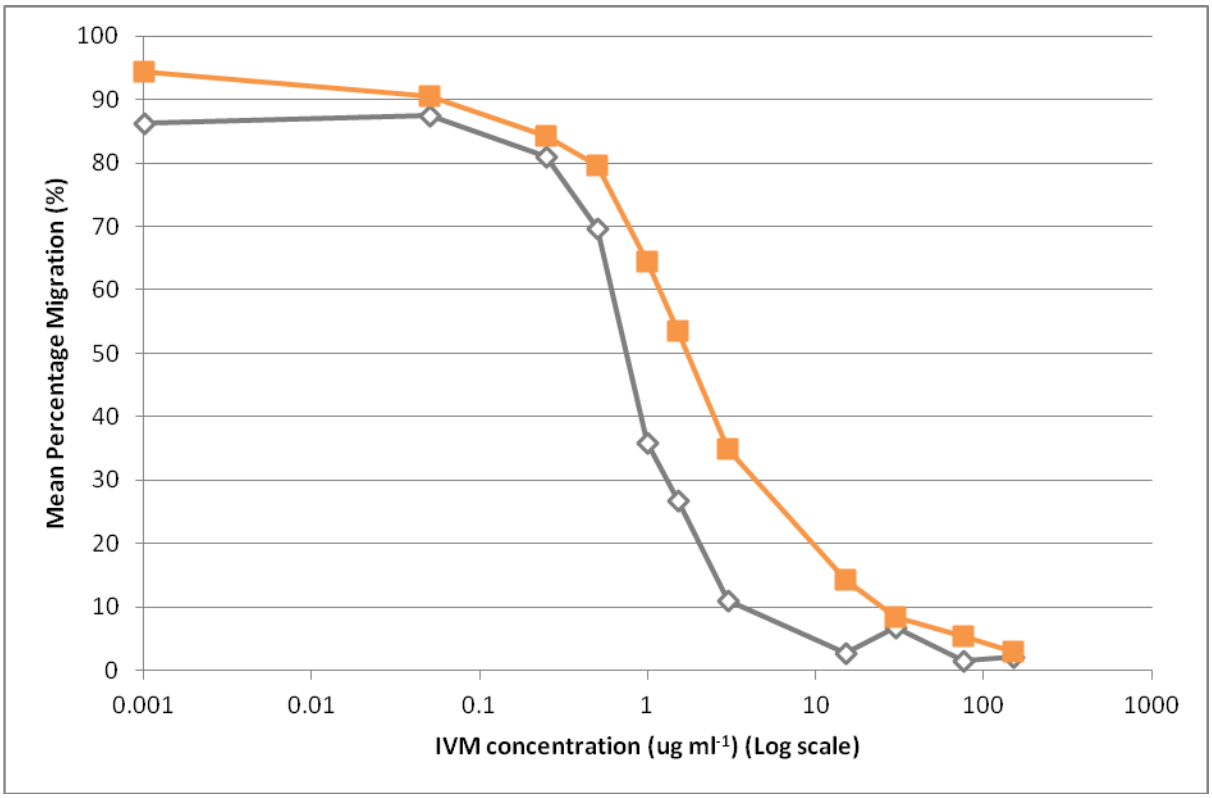

Figure 35: Mean percentage migration of two mixed species field isolates incubated in a range of IVM concentrations. Open and closed symbols represent data derived from FI001 and FI004, respectively. For FI001, the test was conducted five times in duplicate (n=10), and was conducted eight times in duplicate for FI004 (n=16).

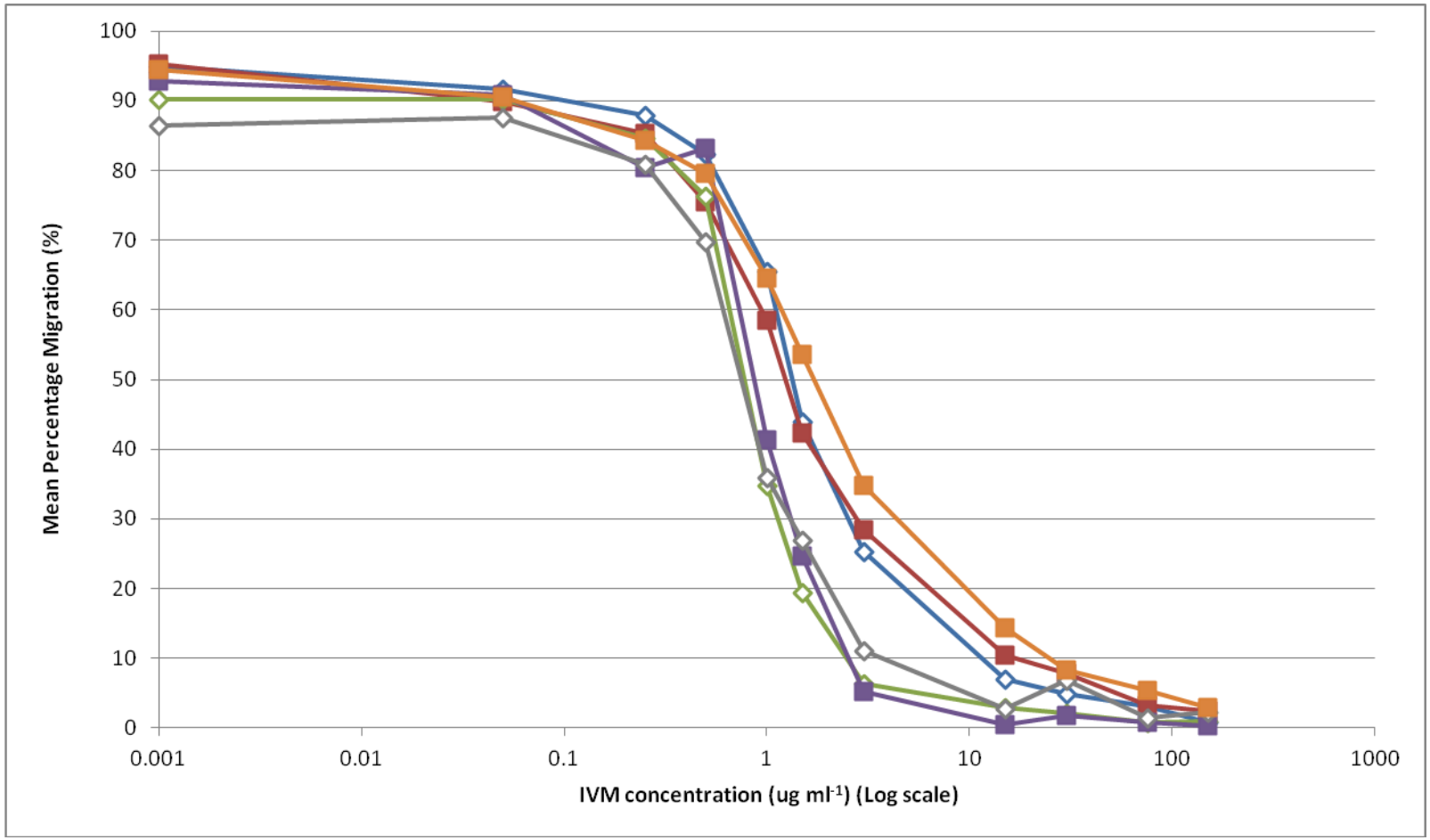

Figure 36: Dose response curves displaying mean percentage L3 migration observed for all isolates examined in the optimised LMIT, across a range of IVM concentrations. Results from Oo IVM-S and Oo IVM-R isolates are represented by blue and red, respectively. Results from Co IVM-S and Co IVM-R are shown in green and purple, respectively, and F001 and FI004 by grey and orange symbols, respectively. All isolates tested a minimum of five times in duplicate.

As shown in Figure 36, migration levels in L_3 derived from all isolates were above 80% migration until exposed to a concentration of 0.25 µg ml^{-1} IVM, when the mean percentage migration began to decrease. As IVM concentration increased, percentage migration of FI001 and both *C. oncophora* isolates (Co IVM-S and IVM-R1) was observed to decrease at a greater rate compared to the *O. ostertagi* isolates and FI004.

Percentage migration values for each isolate were subjected to probit analysis and the resulting LD50 estimates presented in Table 31. The highest LD50 estimate was observed with field isolate FI004 (1.71 µg ml^{-1} IVM), followed by the two *O. ostertagi* isolates (Oo IVM-S, then Oo IVM-R). The other field isolate FI001 provided the next highest LD50 estimate, followed by the IVM-S *C. oncophora* isolate. The lowest LD50 estimate was observed with IVM-R *C. oncophora* L_3 (0.76 µg ml^{-1} IVM). Isolates Oo IVM-S, Oo IVM-R and FI004 were all found to have significantly larger LD50 estimates than each of isolates Co IVM-S, Co IVM-R and FI001, but between these two groups there was no significant difference in LD50 estimates.

Table 31: LD50 estimates generated from probit analysis from all data produced for each isolate

Isolate	LD50 estimate (µg ml^{-1} IVM)	Standard Error
Oo IVM-S	1.53	0.06
Oo IVM-R	1.36	0.08
Co IVM-S	0.78	0.05
Co IVM-R	0.76	0.07
FI001	0.81	0.02
FI004	1.71	0.09

5.4 Discussion

There is a clear need for an *in vitro* test capable of detecting anthelmintic resistance with a high degree of sensitivity and repeatability for field populations of ruminant nematodes (Taylor et al., 2002). With the increase in the number of reports of anthelmintic resistance in cattle nematodes, particularly with regard to ML, such a test could prove to be a useful tool (Sutherland and Leathwick, 2011). Use of a motility test, such as that described by (Martin and Le Jambre, 1979) or a motility meter as used by (Bennett and Pax, 1986; Folz et al., 1987) are not suitable for this purpose due to issues with operator subjectivity or mechanical variability. The ideal *in vitro* test to detect anthelmintic resistance would be: relatively inexpensive; simple to perform; reproducible between laboratories and operators; able to objectively discriminate between resistant and susceptible worm populations regardless of species composition; utilise a life cycle stage that is easily accessible/maintained and be used for an anthelmintic class of relevance and interest to the researcher. The aims here were two-fold; first, to optimise a quantitative LMIT for use with IVM to investigate *O. ostertagi* and *C. oncophora* L_3 ML sensitivity in an adaptation of previously published protocols and second, to utilise the optimised test to compare monospecific and mixed-species field isolates of known anthelmintic sensitivity status.

The first objective was to optimise a protocol using IVM with bovine nematode species L_3 in an adaptation of previously published protocols (Wagland et al., 1992; Rabel et al., 1994; Demeler et al., 2010b). The first evaluation concerned the mesh pore size used to separate live and paralysed L_3. The use of mesh sizes ranging from 20 µm to 100 µm have been published for use with L_3 of various nematode species

(Douch et al., 1983; Sangster et al., 1988; Rabel et al., 1994; Claerebout et al., 1999); however it was decided to focus on 25 µm and 28 µm mesh for bovine L_3 as recommended by (Demeler et al., 2010a). The results from the heat-treatment experiment showed that, with *O. ostertagi* L_3, fewer L_3 'fell through' a 25 µm mesh compared to a 28 µm mesh, and fewer sheathed L_3 'fell through' compared to exsheathed L_3. The width of sheathed *O. ostertagi* L_3 has previously been reported to average 24.8 µm (range 22.5 – 27.5 µm) (Bisset et al., 1984), which correlates well with the report that *T. colubriformis* L_3 have an average width of 25 µm (± 2.1 µm) (Rabel et al., 1994). The width of *C. oncophora* has been published to be 0.03 mm (Isentstein, 1963), although given the larger units used to determine this measurement, this result may not be directly comparable to those observed with *O. ostertagi* or *T. colubriformis*. In addition, the sheaths of *T. colubriformis* and *H. contortus* L_3 have been reported to be 0.55 µm and 0.56 - 0.7 µm thick, respectively (Davey and Rogers, 1982; Wharton, 1986), so it can be supposed that removal of the sheath could decrease larval diameter by 1.1 -1.4 µm in total. If it assumed that sheath thickness is approximately equal across other nematode species, this may account for the slightly higher (although still low) levels of "fall through" observed here with exsheathed L_3. The use of a heat-treatment protocol, rather than killing the L_3 by fixing with ethanol or iodine, prevented any potential shrinkage of fixed worms (as observed whilst enumerating fixed L_3 here), which may have subsequently increased levels of fall through. The high levels of migration observed across all control wells (in excess of 95% migration) suggests that a mesh pore size of 25 µm is not too small to prevent viable larvae from migrating. Even with *C. oncophora*, previously determined to be slightly larger than *O. ostertagi*, mean

percentage migration was still in excess of 91% and 92% in DMSO-only and H_2O-only wells, respectively. It should also be considered that if the pore size was too small to allow *C. oncophora* to fully migrate, there may have been evidence of L_3 becoming caught in the mesh. The migration chamber mesh was examined microscopically after each use and no adhering L_3 were ever observed. As a result of the high levels of migration observed in this optimisation step with *O. ostertagi* and the subsequent migration of *C. oncophora* in the optimised test, a mesh pore size of 25µm was deemed appropriate for use with both *O. ostertagi* and *C. oncophora* L_3.

The next step evaluated was the use of DMSO as a solvent. A previous study highlighted that a major disadvantage of an *in vitro* test compared to an *in vivo* test is the water insolubility of the anthelmintic test substances (Boisvenue et al., 1983). To overcome this, a variety of solvents have been used to dissolve anthelmintic compounds including acetone, ethanol and DMSO (Folz et al., 1987). DMSO has been used in several studies to successfully solubilise IVM (Rothwell and Sangster, 1993; Rabel et al., 1994; Demeler et al., 2010a; Demeler et al., 2010b). To achieve a solubilised solution of IVM, varying levels of DMSO concentration have been tested, ranging from less than 1% total volume (Bennett and Pax, 1986; Sangster et al., 1988) to 5% total volume DMSO (Rabel et al., 1994). Here, high levels of percentage migration (in excess of 90%) were observed across all DMSO concentrations tested, with both sheathed and exsheathed L_3. At 3% DMSO, the mean percentage migration for sheathed L_3 was 99.25% with a mean percentage migration of 97.4% for exsheathed L_3, which was the highest observed with exsheathed L_3 across all concentrations tested. The results presented here with *O.*

ostertagi L_3 suggest that a concentration of 3% v/v DMSO can be used as a solvent, without adversely affecting the migration of sheathed or exsheathed L_3. Furthermore, a concentration of 3% v/v DMSO was also found to dissolve the highest concentration of IVM completely, with no sediment visible after agitation or following incubation, whereas flocculation was observed when DMSO+IVM concentrations (1% v/v) were prepared as described by (Demeler et al., 2010a). Futhermore, the use of 1% v/v DMSO has been reported to give poor solubility of IVM at concentrations greater than 20 µM (17.5 µg ml^{-1}) (Evans et al., 2013). The results presented in this study are also in agreement with those of Rabel et al., (1994), who reported that the use of DMSO up to a concentration of 5% v/v had no adverse effect on L_3 migration. As such, a DMSO concentration of 3% v/v was determined to be the most suitable for the purposes of sufficiently solubilising IVM without consequently affecting the migration of the L_3. As with the mesh pore size evaluation, the consistently high levels of *C. oncophora* migration observed in the DMSO-only control wells of the optimised LMIT confirm that this was an appropriate decision.

Having established appropriate DMSO concentrations and mesh pore size, the next step was to evaluate the difference in migration between sheathed and exsheathed L_3 and if there was any confounding effect of including a Baermannisation step immediately prior to the test. Previous studies have predominantly used exsheathed L_3 in the LMIT (Wagland et al., 1992; Rabel et al., 1994; Claerebout et al., 1999; van Doorn et al., 2010). Some studies, such as those described by Douch et al., (1983), used exsheathed L_3 immediately after exsheathment, whereas other studies

exsheathed larvae the night before LMIT testing (van Doorn et al., 2010). Wagland et al., (1992) claimed exsheathed larvae were able to be stored, if maintained at 4 °C. Sodium hypochlorite has been shown to be lethal to nematodes after prolonged incubation (Rabel et al., 1994). It has also been stated that exsheathed L_3 have a tendency to be more sensitive *in vitro* to anthelmintics compared to sheathed larvae, with (Douch and Morum, 1994) suggesting that differences in cuticle permeability may alter depending on the nematode species being evaluated. The results here would suggest that sheathed *O. ostertagi* L_3 were more susceptible to the effects of IVM, compared to the exsheathed *O. ostertagi* L_3, as shown by the lower LD50 estimate. Larvae observed during the exsheathment process here were visibly more active compared to sheathed larvae and this observation was true for both IVM-S and IVM-R *O. ostertagi* isolates. To ensure that the subsequent LMIT results were not unduly compromised by this observed increase in activity, sheathed larvae were used for all the optimised tests.

Inclusion of a Baermannisation step immediately prior to conducting the LMIT was found to give slightly higher LD50 estimates in L_3 that had been Baermannised compared to those L_3 which had not; however, the difference was not found to be statistically significant. Baermannisation of L_3 prior to use in the LMIT was routinely conducted in studies by Demeler et al (2010a, b). However, previous work has raised concerns about the use of a pre-test Baermannisation step, due to the potential for pre-selecting active L_3 (Glazer and Lewis, 2000), resulting in L_3 having to migrate twice in the course of the test. As a result, it was decided not to include a Baermannisation step in the optimised test here.

In previous studies, the length of time that L_3 are stored for prior to use in the LMIT is widely variable. For example, Geerts et al., (1989) used L_3 that had been stored for less than one month, whereas Demeler et al., (2010a) did not use L_3 that had been stored for more than three months. Results from a study with *T. colubriformis* (Molan et al., 2000) found L_3 previously stored for seven months prior to use were more sensitive to the effect of condensed tannins, compared with L_3 that had been stored for only one month. In an attempt to address the reported inconsistencies of the larval paralysis test (Martin and Le Jambre, 1979), a study by Geerts et al (1989) explored the effect of storage on L_3 stored for one or two months prior to use. MOR-resistant and sensitive *O. ostertagi* isolates were exposed to concentrations of LEV and MOR. When testing the effect of MOR incubation on MOR-resistant *O. ostertagi*, a statistically significant difference in LD50 estimates was observed when comparing LD50 estimates from one month-old L_3 (73.8 µg ml^{-1} MOR) with LD50 estimates generated with two month-old L_3 (27.9 µg ml^{-1} MOR). When a MOR-sensitive *O. ostertagi* isolate was tested with MOR, a similar (albeit non-significant) decrease in LD50 estimates was observed between larvae of different ages: an LD50 estimate of 95.6 µg ml^{-1} MOR was seen with one month-old L_3, compared to the LD50 estimate of 56.1 µg ml^{-1} MOR seen with two month-old L_3 (Geerts et al., 1989). The data from this study where LD50 estimates from *C. oncophora* L_3 stored for six months were compared to LD50 estimates from *C. oncophora* L_3 extracted the previous week showed that there were no significantly differences between the batches of larvae tested (P=0.89). Similarly, with *O. ostertagi* L_3 there was no significant difference between L_3 stored for two months compared to L_3 stored for one week (P=0.39). However, in order to maintain continuity with other studies, and

acknowledging the previous observation that *O. ostertagi* L_3 have the potential to migrate less consistently than other species (Demeler et al., 2010b), L_3were not stored for longer than three months prior to use in the LMIT.

In summary, based on the results generated from the optimisation steps, a final protocol was established using sheathed L_3 that were not Baermannised prior to assessing in the LMIT; a DMSO concentration of 3% was selected to solubilise IVM without having an adverse effect on migration; a mesh pore size of 25 µm and larvae used within three months of being stored at 4 °C.

The second objective here was to evaluate the LMIT for use in determining the IVM sensitivity of a selection of nematode isolates. These isolates had been previously shown to exhibit varying levels of IVM sensitivity *in vivo* and comprised four monospecific isolates and two field (i.e. mixed-species) isolates. For a diagnostic test, capable of detecting the presence of IVM resistance *in vitro*, it can be hypothesised that IVM resistant isolates would exhibit higher LD50 estimates, compared to IVM-sensitive isolates. This would indicate that higher concentrations of IVM are required to paralyse 50% of the resistant population compared to the sensitive population. This hypothesis was not confirmed here.

With the exception of FI004, an inverse relationship was found between *in vitro* LD50 estimates and *in vivo* IVM efficacy data for each isolate. *C. oncophora* present in FI004 had previously been confirmed as IVM-resistant, as described in Chapter 4 and Bartley et al., (2012). Following administration with subcutaneous and pour-on IVM, percentage reductions in small intestinal adult worm burdens (compared with untreated control animals) were 10% and 0% for each treatment,

respectively. In the LMIT, the highest LD50 estimate of all isolates tested was observed when testing L_3 from FI004 (1.71 µg ml^{-1} IVM). If this nematode population was being examined in isolation, the results of the *in vitro* LMIT would appear to correlate well with the *in vivo* CET results. In contrast, the LD50 estimate observed with field isolate FI001 did not reflect the *in vivo* status of the isolate. As previously stated, when a CET was conducted with subcutaneously and topically applied IVM, *C. oncophora* adult worm burdens in comparison to untreated control animals, were reduced by 38% and 64%, respectively, confirming the presence of IVM resistant *C. oncophora* (Bartley et al., 2012). As a result, it could be expected that if the LMIT were capable of detecting IVM resistance, the LD50 estimate of this isolate would be lower than that which was generated with FI004, given the slightly greater efficacy of IVM against FI001. However, the LD50 estimate generated for FI001 was 0.81 µg ml^{-1} IVM; nearly 50% lower than the estimate generated for FI004 and was statistically significant ($P < 0.05$). When L_3 from FI001 were passaged through a lamb to remove *O. ostertagi* L_3 and retain only *C. oncophora* L_3, the LD50 for this isolate (Co IVM-R) was lower still (0.76 µg ml^{-1} IVM). Furthermore, it was lower than the LD50 estimate achieved using Co IVM-S L_3 (0.78 µg ml^{-1} IVM) which had never been exposed to ML compounds (Coop et al., 1979). Similarly, with the two *O. ostertagi* isolates, L_3 from the susceptible isolate (Oo IVM-S) exhibited a slightly higher LD50 estimate (1.53 µg ml^{-1} IVM) than that observed for the laboratory-selected IVM resistant isolate (Oo IVM-R, 1.36 µg ml^{-1} IVM), although this was not a statistically significant difference.

For a diagnostic test capable of detecting IVM resistance *in vitro*, it would be desirable for the ML-resistant isolates to have higher LD50 estimates than the sensitive isolates. With the exception of FI004, this hypothesis does not reflect the findings of this study. Furthermore, *C. oncophora* has been identified as a dose-limiting species for ML anthelmintics, with higher levels of anthelmintic needing to be administered to achieve the efficacy required for product licensing, compared to other nematode species including *O. ostertagi* (Benz and Ernst, 1979; Egerton et al., 1979; Benz and Ernst, 1981; Egerton et al., 1981). Consequently, it could be expected that *C. oncophora* isolates would demonstrate higher LD50 estimates than *O. ostertagi* isolates when tested against IVM *in vitro*, but this was not reflected in the findings here. The results from the ring test described by Demeler et al., (2010a) are in agreement with the findings here, in that an IVM-S *O. ostertagi* isolate was shown to exhibit higher LD50 estimates in the LMIT than those obtained using an IVM-S *C. oncophora* (308 nM IVM, 107 nM IVM, respectively). No IVM-R *O. ostertagi* isolate was available for testing in the previous studies; however, two isolates of *H. contortus* were used (IVM-S and IVM-R) and LD50 estimates from IVM-S *H. contortus* (914 nM IVM) were found to be higher than those exhibited by IVM-R *C. oncophora* (886 nM IVM). When the same protocol was conducted with field isolates (Almeida et al., 2013), the test was able to distinguish between a susceptible *Cooperia* spp. isolate (LD50 estimate 1.2 nmol IVM) and ML-resistant *Cooperia* isolates (LD50 estimates 2.5 - 11.4 nmol IVM). However, the LD50 estimates did not reflect the results of an *in vivo* FECRT, as an isolate where IVM had an efficacy of 52%, showed an LD50 estimate of 6 nmol IVM, whereas an isolate where IVM had 23% efficacy *in vivo*, showed a lower LD50 estimate in the

LMIT (2.5 nmol IVM) and was not found to be significantly different from an IVM susceptible reference isolate (Almeida et al., 2013).

The inverse relationship between *in vivo* and *in vitro* results observed here, and that of (Almeida et al., 2013) has also been reported with other classes of anthelmintics. When LEV-resistant *T. colubriformis* L_3 were assessed in a migration test, the LD50 estimate was often found to be lower than that of LEV-sensitive isolate and LD50 estimates varied from 40 – 300 µM LEV. At the time, the authors considered the test to be too subjective for use in such studies and hypothesised that although adult worms from these isolates were deemed to be LEV-resistant *in vivo*, resistance in L_3 may not be observed phenotypically *in vitro* (Sangster et al., 1988). Similarly, in a study with MOR-resistant *O. ostertagi*, as defined *in vivo*, a higher LC50 estimate was recorded with a MOR sensitive isolate (95.6 µg ml^{-1} MOR) compared to the MOR resistant isolate (73.8 µg ml^{-1} MOR) (Geerts et al., 1989). The authors ventured that this may be due to relatively low levels of MOR-resistance in the 'resistant' isolate; however, a CET reported MOR efficacy of 77% against this isolate, compared to adult worm burdens in untreated animals (Borgsteede, 1988).

For the reasons detailed above, it would appear the LMIT may not be a sufficiently sensitive test for determining levels of ML resistance with mixed-species nematode isolates. There have been few attempts to determine the reasons (or potential mechanisms) responsible for this, either by exploring the differences between *in vitro* and *in vivo* tests or by examining the differences between nematode species and stages being examined. The provenance and species composition of the isolates tested in the LMIT should be considered. Here, the *O. ostertagi* isolates shared a

genetic background, with the IVM-R isolate experimentally selected from the IVM-S isolate (Van Zeveren et al., 2007a). The resulting IVM-R isolate may not necessarily reflect the phenotype or genotype of resistance that may be found in the field as different selection protocols have been shown to produce differing genotypes (Gill et al., 1998; Le Jambre et al., 1999); however at least the close relatedness of the two *O. ostertagi* isolates here allows a reasonable comparison for analysing LMIT data against FECRT results. Likewise, in a study by Gill et al., (1998), *H. contortus* isolates selected using sub-therapeutic IVM treatments in sheep did not display 'resistance' in a number of *in vitro* tests. In contrast, a field isolate of *H. contortus* selected using the recommended dose rate of IVM, did display similar findings *in vitro* compared to *in vivo* (Gill et al., 1998). If this is true for *O. ostertagi*, the phenotype (in this case, the levels of migration) exhibited by the IVM-R isolate may not be necessarily representative of the phenotype of every *O. ostertagi* IVM-R isolate.

Although the IVM-S *C. oncophora* used here was isolated prior to the release of IVM (Coop et al., 1979), the genetic background of this isolate has not been studied. It is supposed that genes conferring for resistance are at very low, levels the first time selection pressure is applied through the use of anthelmintic administration, and that these will increase in frequency with continued treatments (Prichard, 1990). Therefore, it is not known if there are genotypic differences between this unexposed *C. oncophora* isolate and a *C. oncophora* isolate that has been exposed to IVM, but still displays sensitivity to IVM *in vivo*. As with the *O. ostertagi* isolates, this may

mean that the phenotype displayed by Co IVM-S is not representative of all IVM-sensitive *C. oncophora* isolates.

C. oncophora differs from *O. ostertagi* in that it is able to produce a patent infection in both sheep and cattle (Keith, 1953). The IVM-R *C. oncophora* isolate was produced by passaging L_3 from mixed field isolate FI001 through a helminth-naïve lamb, successfully removing the *O. ostertagi* from the isolate. However, previous findings have suggested there are differences within a host species which may give rise to differences between *C. oncophora* L_3 cultured from faeces produced by a lamb and *C. oncophora* L_3 cultured from faeces produced by a calf, when cultured under identical conditions (Isentstein and Porter, 1964). Average measurements of L_3 generated using calves in the Isentstein and Porter (1964) study were found to be slightly longer (909 µm) than L_3 derived from ovine infections (893 µm) but the differences were not found to be statistically significant. The authors proposed that differences in larval length may be a consequence of differences in the host animal's utilisation of diet or differences in the microbiota present in the faeces (Isentstein and Porter, 1964). With these findings in mind, the choice of donor animal species may affect the larvae generated, and so may explain why the LD50 estimate for IVM-R *C. oncophora* isolate (passaged through a lamb) was marginally lower than that of the original FI001. It should be noted that the FI001 isolate contained *O. ostertagi*, which appears to be less sensitive to IVM *in vitro* and so may contribute to this difference between the FI001 and the *C. oncohora* L_3 harvested via sheep infections. FI001 was isolated from a farm which did not farm sheep, and so prior to isolation, was maintained solely through cattle and only passaged through calves once isolated.

FI004 was isolated from a farm which also reared sheep that were co-grazed with cattle. As a result, *C. oncophora* present in FI004 may have been exposed to greater selection pressures for IVM resistance than that of FI001. From the CET data (Bartley et al., 2012), FI004 showed the lowest reduction in adult worm burden with IVM, but was more sensitive to MOX treatment than FI001. Both farms were known to have consistently used ML treatments in calves for the five years prior to performing the FECRT; however, the exact brands used are unknown. Theoretically, use of different anthelmintic products could have resulted in differing selection pressures on each isolate, and as previously demonstrated for *C. elegans*, with differences exhibited in response to IVM and MOX treatment (Ardelli et al., 2009).

Results from a recent LMIT study using filarial nematode L_3 also indicated a disparity between in vivo and in vitro data (Evans et al., 2013). In this study, 30% of ML-sensitive L_3 continued to migrate in IVM concentrations 6000 times greater than that required for 100% efficacy against L_3 *in vivo*. The authors postulated that IVM may be acting on more than one mechanism, which may be responsible for this observed difference and that the host immune system may also contribute to the larval phenotype. The effect of host immunity on parasite behaviour has also been discussed with gastrointestinal nematodes. For example, a study examining egg hatch tests with *H. contortus* found that there were differences in levels of egg hatch dependent on the length of infection (Borgsteede and Couwenberg, 1987). LD50 estimates increased to a peak between 40-60 days post infection, and subsequently decreased after this time. Similarly, Scott et al., (1989) found variation in LD50 estimates when conducting egg hatch tests with *T. circumcincta* eggs, concluding

that differences within host animals may contribute to changes in the LD50 estimates generated (Scott et al., 1989). Consequently, the timing of nematode isolate collection (for example, from newly infected animals or those developing immunity) may also have an effect on the *in vitro* behaviour of L_3.

In vivo and *in vitro* tests examine different life cycle stages and targets may differ between developmental stages. Adult nematodes tested *in vivo* are known to be more sensitive to anthelmintics than the immature stages (Leland Jr. et al., 1975). With respect to IVM, data published from the original efficacy trials showed greater IVM concentrations were required to remove immature stages of *C. oncophora* and *O. ostertagi* compared to adult worms of both species (Egerton et al., 1981). The high levels of IVM efficacy against adult nematodes may be partially attributed to its mode of action, due to the paralysis of the somatic musculature and pharangeal pumping associated with feeding (Kotze et al, 2006). Not all target sites of IVM have been determined; this anthelmintic has been shown to irreversibly bind to glutamate gated chloride channels (GluCl), which increases membrane permeability to chloride ions, resulting in hyperpolarisation of motor neurons and flaccid paralysis of the parasite (Wolstenholme and Rogers, 2005). There are a number of different genes that code for GluCl channels, silencing combinations of these in *C. elegans* can lead to greater tolerance of IVM (Dent et al., 2000). In addition to paralysis of somatic muscle, IVM has also been found to paralyse the pharynx of adult *H. contortus*, the pharynx being the sole muscle of the nematode digestive tract (Geary et al., 1993), and uterine musculature, thus affecting reproductive capabilities (McKellar et al., 1988). It has been hypothesised that a combination of pharyngeal

and somatic paralysis may be responsible for the expulsion of adult worms (Kotze et al., 2012). At lower IVM concentrations, the worms are unable to feed (and so starve) and as IVM concentrations increase, somatic paralysis occurs and worms are unable to maintain their position in the host (Geary et al., 1993; Kotze et al., 2012). As L_3 do not feed, loss of pharyngeal function is unlikely to cause phenotypic response as seen with adult worms and so if both are required to effectively kill adult nematodes, it may partially explain why L_3 appear able to survive higher concentrations of IVM.

GluCl channels are not the only identified targets of IVM in nematodes. Work conducted with *C. elegans* showed IVM can activate gamma amino butyric acid (GABA) activated chloride channel to induced paralysis of nematode musculature (Fisher and Mrozik, 1992). There may be differences between nematode species as the same receptor has been found to be up-regulated in *C. elegans* and down-regulated in *H. contortus,* implying that although receptors may be common between species, they may display different functions (Accardi et al., 2012). There may also be further mechanisms which are unexplored in parasitic nematodes, such as mutations found in the amphid sensory endings of *C. elegans*, associated with cuticle permeability to IVM (Dent et al., 2000). These targets may all differ in expression between species and developmental stages of parasitic nematodes.

Studies of multidrug ABC transporters (predominantly P-glycoproteins, Pgps) in parasitic nematodes have shown that they may play a significant role in ML resistance, as they are cell efflux pumps, and are implicated with limiting drug entry to cells within the parasite (Zhao et al., 2004). Recently, work has been published on

the role of P-gps in ML-resistant *C. oncophora* (de Graef et al., 2013a; Demeler et al., 2013). Differing levels of P-gp expression were found in eggs, L_3 and adult stages. Levels in P-gp expression were also found to differ in adult worms unexposed to MLs and those that survived IVM and MOX treatment, indicating different treatments may also affect P-gp expression. To date, no work has been published on the levels of P-gp expression in *O. ostertagi*, with work in *C. elegans* suggesting P-gp expression is highly diverse and may involve several transporters acting in combination (Lespine et al., 2012).

The complicated nature of IVM resistance, potentially involving a number of mechanisms, means that the use of an *in vitro* test to detect anthelmintic resistance in field populations may not be feasible. However, given the excellent migration in control wells and small standard errors, there could be scope for it to be developed further, in order to examine some of the mechanisms associated with resistance. For example, P-gp inhibitors have been used in a larval feeding inhibition test to restore IVM sensitivity in resistant ovine nematode isolates (Bartley et al., 2009). Use of P-gp inhibitors with IVM-R *O. ostertagi* and *C. oncophora* could give vital information about the mechanisms behind ML resistance in these two species and phenotype within an isolate could be tested in the LMIT.

Based on a variety of parameters, the LMIT was optimised here with a view to being able to be utilised as a diagnostic test for screening mixed-species field isolates for IVM resistance. The results suggest that even an optimised test lacks the characteristic essential for a robust diagnostic test; this was due to incompatibility of the LMIT data with previous *in vivo* results and incompatibility with expected IVM

sensitivities amongst different nematode species. These factors make confirming anthelmintic resistance in populations comprising of a variety of nematode species populations difficult, and possibly beyond the scope of a laboratory-based diagnostic LMIT, as found by a number of other researchers (Demeler et al., 2012; Almeida et al., 2013). However, as mechanisms for ML resistance are still unclear, the LMIT may yet prove to be a useful tool for investigating specific populations of interest to examine the action of particular anthelmintics (Rothwell and Sangster, 1993), or to further investigate unerlying mechanisms associated with resistance (Kotze et al., 2006).

Chapter 6: General Discussion

Anthelmintics are medicinal products, for which, high efficacy is vital to ensure the continued health and welfare of ruminant livestock worldwide. The studies presented in the preceding chapters have considerably added to the knowledge base on gastrointestinal nematode infections in UK cattle and usage of the anthelmintics employed to prevent and control these infections. Exploration of the questionnaire data (Chapter 2) provided an insight into the intense reliance of cattle farmers on macrocyclic lactone (ML) anthelmintics, with the faecal egg count reduction test (FECRT) data (Chapter 3) revealing a predominance of *Cooperia* spp. larvae present following administration of ivermectin on a number of the farms. The examination of different formulae used to determine percentage reduction in FEC was also conducted in Chapter 3. A controlled efficacy test (CET; Chapter 4) confirmed the presence of ivermectin in two of the *C. oncophora* isolates obtained in the field study. This work extended to the further characterisation of these isolates to demonstrate that they were also moxidectin resistant. This was followed by examination of female *C. oncophora* length and oviposition and molecular analysis of a section of glutamate-gated chloride channel (GluCl) gene, (*glc-6*) which had been proposed previously to be down-regulated in ivermectin resistant nematodes following *in vivo* exposure to MLs (de Graef et al., 2013a). Chapter 5 described attempts to develop an *in vitro* diagnostic test for utility in detecting emerging ML resistance in mixed species isolates.

A major finding in this thesis was the presence of ML resistant *C. oncophora* in field populations in combination with ML sensitive *Ostertagia ostertagi*. The observation

of *C. oncophora* surviving ML administration is a now a relatively common finding (Sutherland and Leathwick, 2011). In the field, when the first case of ML resistance is reported in cattle nematodes, the species most commonly implicated is *C. oncophora*; this has been the observation in New Zealand (West et al., 1994); the EU (Coles et al., 1998, Stafford and Coles, 1999); South America (Fiel et al., 2001) and North America (Edmonds et al., 2010). In these studies, ivermectin resistance was defined by FECRT, and, with the exception of the New Zealand study in which species was confirmed by faecal culture, ivermectin resistance in *C. oncophora* was confirmed via controlled efficacy trials in housed cattle. As discussed in Chapter 3, *C. oncophora* is one of the dose-limiting parasites for ivermectin, and so the results here were not surprising (West et al., 1994). Although sensitivity of the *Cooperia* isolates to other anthelmintic classes were not tested here, studies in New Zealand, where all 56 farms tested were found to have ML resistant *Cooperia* spp., also identified that 74% of these farms also had albendazole resistant *Cooperia* (Waghorn et al, 2006). The finding of resistance in more than one nematode species has been observed in the USA, where ML- and benzimidazole-resistant *Cooperia* spp. were identified together with ML-resistant *Haemonchus* spp. (Gasbarre et al., 2009). Worryingly, a controlled efficacy test in the USA confirmed a 0% reduction in adult *C. oncophora* at 14 days after IVM administration and a 90% reduction in adult *O. ostertagi*, the first confirmed case of ML-resistant *O. ostertagi* in the world (Edmonds et al. 2010). As a result, it could be proposed that the observation of ML resistance in *Cooperia* spp. can be considered as a precursor to anthelmintic resistance being detected in other nematode species. Thus, steps need to be taken to reduce further selection for resistance by altering farm practices.

Currently, there are no therapeutic alternatives to using one of the three anthelmintic classes licensed for use in cattle in the UK. Two anthelmintic products launched for use in sheep in the last 5 years, monepantel, an amino-acetonitrile derivative (Kaminsky et al., 2008), and a combination product, comprising abamectin and derquantel (Leathwick and Hosking, 2009) have not been registered for use in cattle. This may be, in part, due to relatively low efficacy of monepantel (78%) observed against adult *Dictyocaulus filaria*, the ovine lungworm (Hosking, 2010). It has also been proposed that other factors, such as the costs required for product synthesis or compliance with safety guidelines, may be a barrier to development of products for species other than sheep (Epe and Kaminsky, 2013). Even if these products were to be released immediately for use in cattle, the issue of anthelmintic resistance will remain. Resistance to monepantel has already been recorded in goats in New Zealand, where resistant *Teladorsagia circumcincta* and *Trichostrongylus colubriformis* have been identified (Scott et al., 2013). As a result, the emphasis must be to preserve the anthelmintics that are currently available for cattle.

The findings from the research described in Chapters 2 and 3 indicated that farmers are often constrained by availability of land, stocking densities, lack of time/handling facilities, and weather conditions. Prior to this research, little was known about which anthelmintic classes were used and the application methods applied on UK cattle farms. A small scale survey (72 questionnaires) had been conducted previously on beef cattle farms to examine anthelmintic products usage (Barton et al., 2006), but did not enquire about quarantine or management procedures, or use of faecal egg count (FEC) analysis. Similarly, few details had been published detailing

FECRT analyses in cattle, with the majority of reports regarding reduced efficacy limited to case reports (Coles et al., 1998; Sargison et al., 2009; Orpin, 2010; Sargison et al., 2010). To date, no analysis had been conducted to examine the relationship of management practices to anthelmintic efficacy across farms. In Chapters 2 and 3, the questionnaire data revealed that beef and dairy farmers used similar numbers of anthelmintic treatments as first season grazing calves were administered with anthelmintics, on average, twice per year, with over 80% of farmers using ML products. In terms of differences identified between the two sectors, dairy cattle farmers were significantly less likely to isolate new stock following quarantine treatment and were less likely to attend farmers meetings relating to parasitology or utilise FEC analysis. Only one respondent had conducted a FECRT prior to this study, demonstrating a clear need for promoting further testing and monitoring of FEC. The benefit of making use of regular FEC analysis is that farmers can gain an understanding about levels of nematode contamination on their pastures, and so could use this knowledge in pasture management strategies, perhaps by grazing other animals, such as sheep on heavily infected pastures, and in combination with FECRT to ensure that the anthelmintics they are using are actually effective. Farmers also tended to apply long-acting anthelmintics to first season grazing calves at turn-out and again at housing, but due to a lack of FEC monitoring, were potentially unaware of levels of pasture contamination from nematodes passed out during the course of grazing season. By promoting FEC analysis in determining treatments, applications may be targeted, particularly in older calves and cows who may have developed immunity to reinfection, and so may only require a single treatment at housing to target inhibited *O. ostertagi* larvae. First season grazing

calves from the dairy sector, which are not grazed with their dams, are more likely be exposed to higher levels of worms on pasture over the grazing season and so closer monitoring of FECs in these calves throughout the season could track levels of pasture contamination, allowing for intervention with anthelmintics when FEC levels are high.

Given the lack of appropriate quarantine activities across the dairy sector farms here, this should be promoted more within the industry (Brennan and Christley, 2012). Current UK quarantine guidelines recommended in the Control of Cattle Parasites Sustainably (COWS) publication suggest that sequential administration of an oral benzimidazole product and a product containing levamisole (LEV) should be used when new cattle are brought onto farm. However, given conflicting data regarding efficacy of fenbendazole against inhibited *O. ostertagi* larvae and lack of an efficacy claim for LEV against these stages, it is possible that inhibited larvae may survive treatment. A further complication is that in the UK, there are few anthelmintics with a short withdrawal period licensed for use in milking dairy cattle. There are no LEV products licensed for use in dairy cattle and withdrawal periods for BZ products vary between 60 hours and 120 days (VMD database, accessed August 2014). Both factors may go some way to explain the limited implementation of quarantine procedures in dairy cattle and the predominance of ML products used in the cattle industry, both as quarantine administrations and for general control.

The questionnaire analysis demonstrated a general lack of accordance with the COWS guidelines, which include: adopting quarantine measures, selecting an appropriate product and ensuring it is applied correctly, devising a parasite control

plan with an advisor, using anthelmintics only when necessary, testing anthelmintic efficacy, reducing dependency on anthelmintics and using methods to preserve susceptible nematodes (COWS, 2010). The questions that arise from these findings are; first, are farmers aware of these guidelines and, second, as veterinary surgeons were deemed to be the most important source of advice, are they making sufficient efforts to promote responsible anthelmintic use? For example, veterinary practices or prescribing premises could arrange meetings for farmers or promote best practice through newsletters. Another factor that could affect uptake of best practice guidelines is the practicality of the COWS recommendations, as shown in the example of quarantine treatments above. It should also be noted that the currently available COWS manual is a large document, spanning 64 pages, which could be off-putting for farmers and advisors seeking clear and accessible advice. Further consultations with farmers may be helpful to ascertain the feasibility of following the current guidelines on farm.

Of the 20 IVM FECRTs performed (Chapter 3), IVM resistant *Cooperia* were identified in samples from over 50% of the farms. These results are similar to those reported previously (Waghorn et al., 2006; Soutello et al., 2007; El-Abdellati et al., 2010a). The FECRT outputs were subsequently analysed with respect to the questionnaire survey responses to explore risk factors for resistance, and a number of differences were found between farms with IVM-sensitive *Cooperia* spp., and those with IVM-resistant *Cooperia* spp. The most striking observations were differences in the management of adult cows. On 40% of farms where IVM-resistant *Cooperia* were detected, adult cattle were given anthelmintics at housing; however, this

practice was highlighted by only 16% of farmers where IVM-sensitive nematodes were identified. In addition, on 46% of farms where IVM resistance was detected, farmers did not treat adult cattle on a selective basis and administered anthelmintic to all adult cows, a practice conducted on only 16% of farms where IVM sensitive nematodes were identified. Although the type of farm could not predict the presence or absence of anthelmintic resistant nematodes, beef cattle farmers were found to be more likely to follow specific recommendations highlighted in the current best practice control guidelines, for example, administering anthelmintic according to the heaviest animal in the herd and isolating new cattle brought onto farm. This may be because in this study, the beef farmers were more likely to farm sheep, with 69% of respondents also farming sheep, a sector in which the Sustainable Control of Parasites in Sheep (SCOPS) guidelines (National Sheep Association) have been disseminated for many years (Abbott et al., 2005). These results highlight that there is need for a greater awareness of evidence-based helminth control in the cattle industry, particularly in the dairy sector. It could be argued that the relatively low numbers of respondents here may have skewed the results of the odds ratios; however, conservative precautions were taken during this analysis as odds ratios with confidence limits spanning 1 were discarded from the analysis (Chapter 2). An interesting follow-up to this study may be to repeat the questionnaire after the FECRT analysis had been conducted and the results disseminated, to see if attitudes have changed in the intervening years.

The finding that only one of the questionnaire respondents had previously undertaken a FECRT is indicative of a general lack of engagement regarding the threat of

anthelmintic resistance. This is further hindered by lack of a clear, standardised method for FECRTs in cattle, and this might act as a disincentive to veterinary surgeons to offer these tests. FECRT guidelines for cattle are undergoing required development (Sutherland and Leathwick, 2011). The work in Chapter 3 demonstrated that a robust FECRT method requires sensitive FEC methodology. When conducting FECRT in the field it is impossible to know if there is 'true' inefficacy or if this is an artefact of misadministration. Confirmation of two of the FECRT results here using the CET substantiated that the FECRT method employed in Chapter 3 was robust. This was further confirmed by analysis of the farms identified as having IVM sensitive nematodes using one calculation method, in the most part, being classified as IVM sensitive using other analytical methodologies. Regardless of the formulae used in calculating thresholds for efficacy, FECRT analysis must be underpinned by good practice in terms of administering the products, handling and storing the samples and using a FEC method of the appropriate sensitivity, as made apparent here by the comparison between the McMaster method (Gordon and Whitlock, 1939) and the double centrifugation method (Jackson, 1974) in estimating FECs. Areas for further work could include testing further anthelmintic classes at these sites, particularly to test if there is insensitivity to BZ or LEV products. WAAVP recommendations (Coles et al, 1992,) suggest a non-treated control group per test; however, on many of the farms here, the first grazing season group sizes were too small and splitting of the groups would have rendered the results statistically less meaningful. Also, a main objective of anthelmintic administration at housing time, when the studies here were performed, is to kill inhibited *O. ostertagi* larvae, thus preventing Type II ostertagiosis, so there

would have been ethical considerations regarding withholding this treatment. As most samples were taken just prior to winter housing, samples taken earlier in the year may have had different nematode genus compositions (Michel, 1969), which may have affected the efficacy observed. As the majority of farms participating here did not handle their first season grazing calves over the summer grazing season, faecal samples could not be taken prior to the autumn.

This study provided this first report of MOX resistant *C. oncophora* in UK cattle. The direct comparison between ML application methods (injectable and pour-on) in UK cattle was also novel. Monitoring FEC through a longer period of the CET may have shown if the observed suppression in egg production was a temporary effect, and would have allowed direct comparison to the on-farm FECRT data; however, the decision was taken to necropsy seven days after anthelmintic administration, exactly as recommended by the WAAVP guidelines (Wood et al., 1995). Examination of nematode length and numbers of eggs *in utero* was examined here to analyse the phenotype of the parasites surviving ML treatment in comparison to nematodes from the untreated control calves. Few studies have been published to compare worm length and eggs in utero in cattle nematodes in anthelmintic treated animals, but these phenotypic markers have been compared more often through the course of natural or experimental infections designed to monitor the development of immunity in cattle (Kloosterman, 1971; Albers et al., 1982; Kloosterman et al., 1991). Recently, a study in Belgium examined *C. oncophora* eggs in female worms unexposed to anthelmintic administration and identified an average of 68 eggs per nematode (de Graef et al., 2012). This finding is similar to the findings of here and

earlier studies in untreated animals (Albers et al, 1982, Kloosterman 1971. The difference in number of eggs *in utero* here was seen to differ between ML products, and between application methods. The absence of any eggs in nematodes from either isolate following MOX administration indicates a longer egg suppression effect with this product compared to IVM, indicating that the anti-parasite effects of ML anthelmintics vary between compounds.

Whilst the FECRT and CET analyses provided vital data regarding anthelmintic sensitivity of the isolates examined here, time, cost and labour resource that needs to be invested in these types of experiments indicates that the development of *in vitro* tests would be beneficial. With regard to further analysis of the isolates (FI001 and FI004) characterised here, the larval migration inhibition test (LMIT) analysis indicated discrepancies between *in vitro* and *in vivo* ML sensitivity classification, an observation similar to other recent studies (Whitney et al., 2011; Demeler et al., 2012; Almeida et al., 2013). Earlier studies with the LMIT using single nematode isolates yielded reproducible dose-response curves which appeared to be in agreement with anthelmintic sensitivity classification derived from *in vivo* studies (Wagland et al 1992, Rabel et al 1994). Even with single species populations here, the relative degree of ML sensitivity did not concur with the observed *in vivo* sensitivity of the isolates; for example, ML-sensitive *O. ostertagi* isolates displayed higher resistance ratios than ML-resistant isolates of *C. oncophora*. The latter observation is in agreement with those of Demeler et al (2012).

To underpin the results of the *in vivo* testing and to aid further development of *in vitro* tools, an understanding of the mechanism(s) of ML resistance at the molecular

level would be beneficial, particularly for screening nematode populations in the early stages of resistance. A glutamate-gated chloride channel subunit (glc-6) gene was previously been shown to have a SNP in the signal peptide region of the gene in a ML-exposed, ML-resistant *C. oncophora* isolate (De Graef, 2013). The peptide sequence associated with this spanned an aspartic acid (D) change to glycine (G). However, this SNP was not identified in any of the peptide sequences analysed from male and female nematodes from isolates FI001 and FI004; either from nematodes recovered from untreated control animals or from nematodes that had survived injectable IVM administration. There were no differences in amino acid sequence observed between ML resistant isolates here and a previously generated sequence from a Belgian ML-sensitive isolate (de Graef et al., 2013a), indicating this SNP may not be involved in ML-resistance in all *C. oncophora* isolates. It would have been beneficial to have access to a ML-naïve parental isolate of both ML-resistant *C. oncophora* isolates, and to examine sequences in nematodes surviving MOX administration. Future studies to investigate this further could include the examination of genome sequences from single worms, through the exploration of next generation sequencing or deep-sequencing, as the capability of these technologies increase. For example, in a recent study on *Dictyocaulus viviparus*, (Cantacessi et al., 2011) a large-scale examination of transcriptome data across several life-cycle stages using 454 sequencing yielded 8-fold greater coverage than a study conducted in 2007, which utilised a conventional sequencing approach (Ranganathan et al., 2007). Previously, studies detailing the transcriptome of *O. ostertagi*, (Abuker et al., 2009) and *C. oncophora* (De Graef, 2013) have been published, compiling thousands of expressed sequence tags (ESTs) (Abuker et al.,

2009). However, with the use of 454 sequencing, greater coverage of both transcriptomes have recently been published (Heizer et al., 2013). This knowledge could be vital in identifying genes which are associated with resistance developing across all anthelmintic classes..

Whilst the phenotypic and genotypic analyses here did not reveal a diagnostic tool or marker for ML resistance in cattle nematodes, the information could be exploited to explore a narrower range of anthelmintic concentrations in the LMIT across the range where the biggest change in migration occurred (around at 1 μg ml^{-1} IVM), and larvae that migrate or not could be subjected to analysis to examine changes in gene expression proposed to have a role in ML resistance, such as the P-glycoproteins (de Graef et al., 2013b; Demeler et al., 2013). There is also scope to use P-gp inhibitors to examine the potential for these compounds to reverse ML resistance *in vitro* (Bartley et al., 2009). For example, in *Haemonchus placei*, P-gp inhibitors were successful in improving *in vitro* IVM efficacy against IVM-resistant *worms* and were able to reduce the effective concentration (EC50) estimate with a number of inhibitors, compared to IVM alone (Heckler et al., 2014). To explore this work further, a molecular test could be added to the end of the *in-vitro* test to examine larvae which either migrated or fed, or failed to do so and the genetic differences between these populations, for example in the up- or down-regulation of different Glu-Cl subunits or P-gps.

Taking the entire work of this thesis into account, the most important areas requiring further research are clear. Whilst the most straightforward approach would be to conduct more FECRT, using different anthelmintic classes and gather more data

from farmers to identify their parasite control practices, there is a general lack of information regarding the epidemiology of these parasites and the impact of anthelmintic selection. The majority of epidemiological data in the UK was published in the 1960s and 1970s, i.e. before registration of most broad spectrum anthelmintics currently in use (Michel, 1963, 1968, 1969a, b,; 1970, 1976; Armour et al., 1969a, b; Michel and Sinclair, 1969, Michel and Lancaster, 1970; Michel et al., 1970, Armour, 1970, 1974; Michel et al, 1978; Michel et al., 1978b) and so there is little information regarding the changes in nematode populations that may have occurred due to extensive use of broad spectrum anthelmintic products, in particular the MLs. With the likely shift in nematode genus composition following ML administration, *Cooperia* spp. could dominate cattle nematode populations. Worryingly, in the course of a small-scale CET, it was previously reported that an ML-resistant *C. oncophora* isolate was more pathogenic than a non-related susceptible isolate (Njue and Prichard, 2004c). Two calves infected with the resistant isolate suffered from ill-thrift and signs of parasitic gastroenteritis, which was not observed in two calves infected with the same number of larvae from the ML-susceptible isolate (Coles et al., 2001; Njue and Prichard, 2004c). Due to the small numbers of calves used, there is a need for further work in this area, especially with isolates that are more closely related, to prevent other population differences confounding the results. This is not only pertinent to cattle farmers, but those who co-graze sheep with cattle, given the ability of *C. oncophora* to infect both species (Isenstein, 1963; Isenstein and Porter, 1964). Further work on such farms could investigate the effect of ML applications given to sheep as this, in theory, would provide additional ML selection upon *C. oncophora* or *Trichostrongylus axei*

nematodes present. Future work should take the whole herd into account to ensure confounding variables can be accounted for. Such an example, would be additional ML applications to treat psoroptic mange (Mitchell et al., 2012), which will add additional selection pressure to nematodes, as has been reported in the sheep industry in relation to long acting ML treatments for *Psoroptes ovis* (Taylor, 2012). Again, to spread awareness about this issue in the cattle industry, veterinary surgeons and advisors must collaborate with their clients to ensure anthelmintic applications are justified.

6.1 Summary

By combining parasitological knowledge with knowledge of current farming systems optimum anthelmintic use can be achieved, whilst maintaining productivity and preserving efficacy of the current anthelmintic classes for as long as possible. Knowledge regarding the epidemiology of the parasites in the current management strategies and climatic conditions is vital, and encouragement to adopt "best practice" principles must be continued through efforts of veterinary professionals and industry advisors through a variety of accessible media. Strategies need to be feasible to encourage uptake of advice and to aid farmer compliance, but they must also be underpinned by strong empirical evidence, which is currently lacking in the dairy and beef cattle sectors. With no new classes of anthelmintic likely to appear on the market in the short to medium term for cattle, there is an urgent need to understand the mechanisms behind anthelmintic resistance, (Sutherland and Leathwick, 2011). If, or when, these mechanisms are discovered, new tests (for

example, cheap molecular screening tests) could be used to support advice and deployment of strategies aimed at reducing dependence on anthelmintics. Changes in marker frequency could be indicative of resistance developing before any phenotypic effect could be discerned (Ellis et al., 2011; Jackson, 2013). As a result, the early detection of anthelmintic resistance could be dramatically improved and this information integrated into evidence-based decision support systems for farmers and livestock producers.

Appendix 1: Questionnaire Survey

Cattle Parasite Management Questionnaire 2012

For many years information regarding views and approaches to worm control by UK sheep producers has been collected and analysed, partly stimulated by the desire to better understand how anthelmintic resistant parasites may be selected for and spread and to provide advice on how best to combat it. In cattle the situation is less clear and the aim of this questionnaire is to identify what approaches are used by farmers in the management and treatment of worm infections. We would ask cattle producers to kindly take a few minutes to complete this questionnaire and return FREEPOST to the address overleaf. **All data will be treated as confidential.**

1. Farm name?			2. Post code? ……	
3. How long have you owned/rented the farm?				
4. Total area of grazing pasture?	…… Acres or …… Hectares How much is permanent pasture? ……			
5. Herd size - adult cattle (> 24 months)? - stirks (1 - 2 years)? - calves (up to 1 year)?	Beef		Dairy	
6. What are your average stocking rates per hectare?	Adults	Less than 3: ☐	3 - 5: ☐	More than 5: ☐
	Stirks	Less than 5: ☐	5 - 10: ☐	More than 10: ☐
7. Are sheep also kept on the farm (please give annual figures)?	No: ☐	Ewes: …….	Lambs: …….	Tups: …….
8. What months does calving occur on your farm?	State which months:			
9. Is grazing area for -	Sheep only: ☐	Cattle only: ☐	Sheep & cattle: ☐	
10. When did you start grazing last year? Please state which month.	Adults:	Stirks:	Calves:	
11. When did you end grazing last year? Please state which month.	Adults:	Stirks:	Calves:	
12. How long do you graze animals per day (hours)?	Adults:	Stirks:	Calves:	
13. Did any of the age groups graze together?	Please give details.			
14. Do you change grazing pasture?	Yes: Within a grazing season ☐ Yes: Every 1-2 years ☐	No: ☐		
15. Do you "usually" give supplementary feed (hay/silage/concentrates)?	Yes: Spring ☐ Yes: Autumn ☐	No: ☐		
16. Do you move your calves to new pasture after weaning?	Yes – Clean grazing: ☐ Yes – Dirty pasture: ☐	No: ☐		
17. Do you co-graze, rotationally graze or graze your animals separately?	Graze cattle: Cattle only enterprise:☐ Separately: ☐	Rotationally graze: Cattle & Sheep: ☐ Cattle & Goats: ☐	Co-graze: Cattle & Sheep: ☐ Cattle & Goats: ☐	
18. Do you mow your pasture before or during the grazing season?	Yes, most of the pasture was mowed (over 50%): ☐ Yes, some of the pasture was mowed (less than 50%): ☐	No: ☐		
19. Do you introduce new stock onto farm?	No: ☐	Adults: ☐	Stirks: ☐	Calves: ☐
20. How often do you buy animals?	Less than annually: ☐	Annually: ☐	More often: ☐	
21. Where do you purchase your new stock from?	Market: ☐	Auction: ☐	Private sale: ☐	Other ☐
22. Do you deworm animals brought onto the farm? See attached sheet for a list of available treatments and their classes	No: ☐	Yes, with: Class 1 ☐ Class 2 ☐ Class 3 ☐		
23. Do you isolate stock after quarantine treatment? Please state length of isolation.	No: ☐	Yes: ☐ …… days		
24. Do you routinely treat your animals with anthelmintic? If not what do you do instead?	Yes: ☐	No: Organic ☐ Use grazing management ☐		
25. How often do you deworm your calves- numbers of times and which months of the year? Circle the appropriate month(s).	Number of times per annum:			
	Beef: 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 6+☐			
	Dairy: 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 6+☐			
	Months - Circle the appropriate month(s).			
	Beef: J F M A M J J A S O N D			
	Dairy: J F M A M J J A S O N D			

Continued Overleaf

26. Do you feel worms are a problem on your farm?	Yes, minor ☐ Yes, moderate ☐ Yes, serious ☐			No: ☐			Unsure: ☐				
27. How do you deworm your animals? (Tick all that apply)		Cows	Heifers	Calves							
	Follow a set drench program	☐	☐	☐							
	At sign of disease	☐	☐	☐							
	At housing	☐	☐	☐							
	At turning out	☐	☐	☐							
	Weaning	☐		☐							
	Mating	☐									
	Pre/post calving	☐									
28. What class of anthelmintic(s) did you use in the last 12 months?	Class 1 (Benzimidazoles) ☐	Class 2 (Levamisole) ☐	Class 3 (Macrocyclic lactones) ☐								
29. What form of anthelmintic do you use to treat your cattle? (Tick all that apply)	Bolus: ☐	Injectables: ☐	In feed: ☐ Pour-on: ☐	Drenches: ☐							
30. If using an oral drench do you check the accuracy of the dosing equipment?	No: ☐	Yes: ☐									
31. How long have you been using present drug?		1 Year	2 Yrs	3 Yrs	4 Yrs	5 Yrs	5+ Yrs				
	Beef:	☐	☐	☐	☐	☐	☐				
	Dairy:	☐	☐	☐	☐	☐	☐				
32. What anthelmintics have you used in the past 5 years?		Adults					Calves				
		'09	'08	'07	'06	'05	'09	'08	'07	'06	'05
	Class 1	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
	Class 2	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
	Class 3	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
33. How often do you change your anthelmintics?	Never: ☐	Each time you use drench: ☐ Every 3 months: ☐ Every 6 months: ☐ Annually: ☐ Longer: ☐									
34. Do you deworm all of your animals or do you deworm selectively?	Deworm selectively: ☐	Yes, all: Adults ☐ Stirks ☐ Calves ☐									
35. How do you determine amount of dewormer to use?	Estimate weightof each animal: ☐ Dose to average weight of herd: ☐ Weigh & dose to heaviest animal: ☐ Weigh individual animals and dose accordingly: ☐										
36. Do you ever withhold food before deworming?	No: ☐	Yes: ☐ (How long before?)									
37. Do you use anthelmintics with additional Cobalt/Selenium?	No: ☐	Yes: ☐									
38. Where do you find information about anthelmintics & deworming programs? (Tick all that apply) 39. Rank the three most importance sources, with 1 as the most important and 3 the least.		Rank									
	Journals/magazines ☐										
	Advertisements ☐										
	Meetings ☐										
	Veterinarians ☐										
	Other farmers ☐										
	Pharmaceutical Reps ☐										
	Other (Please specify) ☐										
40. How effective do you think your dewormers are in comparison to previous years?	More: ☐	Less: ☐	Unaltered: ☐								
41. Do you move your animals to clean pasture after treatment?	No: ☐	Yes: ☐									
42. Do you have a problem with any other parasites?	No: ☐	Yes: Liver fluke ☐ Tapeworm ☐ Mites/Lice ☐ Warbles ☐ Lungworm ☐									
43. Do you monitor faecal egg counts?	No: ☐	Yes: Occasionally ☐ Yes: routinely ☐									
44. Have you tested for drug resistance?	No: ☐	Yes: ☐									

Thank you for taking the time to complete this questionnaire.

Please return FREEPOST to - Dr D. Bartley, The Moredun Foundation,
FREEPOST EH1686, PENICUIK, EH26 0FG.

Appendix 2

Results from Farms 001 - 004 presented in Chapter 3 have previously been published in a peer-reviewed short communication: McArthur, C.L., Bartley, D.J., Shaw, D.J., Matthews, J.B. (2011) “Assessment of ivermectin efficacy against gastrointestinal nematodes in cattle on four Scottish farms.” *Veterinary Record* 169, 658.

Short Communications

Assessment of ivermectin efficacy against gastrointestinal nematodes in cattle on four Scottish farms

C. L. McArthur, D. J. Bartley, D. J. Shaw, J. B. Matthews

CURRENT control strategies targeted against gastrointestinal nematode infections in cattle rely heavily on the use of anthelmintics. Three drug classes are licensed for this purpose in the UK: benzimidazoles, imidazothiazoles and macrocyclic lactones (ML). The latter, in particular, ivermectin (IVM), is used extensively primarily because of its high efficacy and wide safety indices (González Canga and others 2009). Anthelmintic resistance (AR) has been reported widely in nematodes of small ruminants, but there have been fewer reports in cattle. The reason for this may be due to a true lower incidence of AR, because cattle are generally 'drenched' less frequently than sheep. It may also be due to the fact that infections caused by *Cooperia oncophora*, for which AR has been most commonly recorded in cattle, may not be detected because of the relatively low pathogenicity of this nematode in cattle. However, recently, there has been an increase in reports of AR in cattle, especially in the southern hemisphere (Sutherland and Leathwick 2011). Single populations have been identified that are resistant to multiple anthelmintic classes (Waghorn 2006).

The first UK case of IVM resistance in cattle nematodes was found in 1999 (Stafford and Coles 1999), and other reports have been published subsequently (Sargison and others 2009, Orpin 2010). Apart from these studies, little research has been undertaken on the regional prevalence of AR in cattle nematodes in the UK. A questionnaire study of helminth management practices on Scottish cattle farms is underway at the Moredun Research Institute. As part of this, farmers have been asked to participate in a faecal egg count reduction test (FECRT) to assess the efficacy of IVM on their farm. Here, data are presented from the first four farms that participated in these IVM-FECRTs.

Three farms were sole beef producers and the other comprised dairy and beef cattle; all farmers had used ML treatments, on average, twice per annum over the preceding five years. The farms were located in Ayrshire, Dumfriesshire, Orkney and the Scottish Borders, and the FECRTs were conducted over a six-week period during October and November 2010. Participants were issued with a sample kit containing specific instructions on how to conduct the FECRT, injectable IVM (Ivomec Super; Merial Animal Health), sample bags, weight tapes, needles and syringes. Participants were asked to identify and sample 10 to 15 first-season grazing (FSG) calves. On the day of treatment (day 0), animals were weighed and a manufacturer-recommended dose rate of 0.2 µg IVM/kg bodyweight was administered subcutaneously. Farm 1 used a weight tape to weigh calves, whereas farms 2, 3 and 4 used weigh crates. Information regarding weight data (obtained using crate scales) and doses administered was volunteered from farms 2 and 4. Freshly passed faecal samples were collected into ziplock bags (Gripwell), as much air as possible was excluded and the samples were sent Freepost to the Moredun Research Institute. Samples obtained at day 0 and day 14 post-treatment were subjected to faecal egg count (FEC) analysis by the following method. Faeces were mixed thoroughly by homogenisation, and a 10 g subsample was taken from each. Subsamples were mixed thoroughly in 100 ml tap water. FECs were conducted in duplicate for each sample by removing two 10 ml aliquots and using a modification of the salt flotation method described by Jackson (1974), which has a sensitivity of 1 egg per gram (epg). Further material was taken from each sample for screening for *Dictyocaulus viviparus* first-stage larvae (Ministry of Agriculture, Fisheries and Food 1986) and for *Fasciola hepatica* eggs (McCaughey and Hatch 1964). Equal quantities of faeces from all animals from farms 2, 3 and 4 were pooled for coproculture and incubated at 22°C for 14 days to provide third-stage larvae (L_3) for morphological identification of L_3 in both pre- and post-IVM treatment samples. Because of the small sample sizes submitted, all excess faeces from animals in farm 1 were pooled and cultured. Morphological identification of L_3 to genus level (Ministry of Agriculture, Fisheries and Food 1986) was conducted on 100 randomly selected L_3 per sample at x100 magnification.

Farms 1 to 3 provided samples from a single group of FSG calves grazing at foot with their dams, whereas farm 4 provided samples from two cohorts of FSG calves (groups 4A and 4B): calves in group 4A had been turned out with their dams earlier in the season than calves in group 4B, which were grazed without their dams. Overall, the day 0 trichostrongyle FECs from all four farms ranged from 0 to 225 epg, with post-treatment FECs ranging from 0 to 52 epg (Table 1). *F. hepatica* eggs and *D. viviparus* larvae were not observed in any pre or post-treatment samples. Current guidelines for assessing anthelmintic efficacy in cattle are less well defined than those for sheep, and state that a minimum individual FEC should be at least 100 epg for inclusion of individual animals in the test (Coles and others 2006). However, the recommendations also state that 'if initial egg counts are below 150 epg, egg counting may require the use of a method more sensitive than the modified McMaster technique used for sheep' (Coles and others 2006). Although some of the day 0 FECs were less than 100 epg, the technique used here had a sensitivity of 1 epg. In the 1992 World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines, percentage efficacy thresholds were set for diagnosing AR in the ruminants: for ML anthelmintics, these were quoted as a mean FEC reduction of less than 95 per cent, with a lower 95 per cent CI of less than 90 per cent (Coles and others 1992). Using this classification, farms 1, 3 and 4 would be categorised as harbouring IVM-resistant nematodes, because a FEC reduction of less than 95 per cent in epg was achieved at each site (Fig 1). Morphological analysis of L_3 (Table 1) indicated a high proportion of *Cooperia* species in the samples obtained before and after treatment. This finding is consistent with other studies conducted in northern Europe (Demeler and others 2009, El-Abdellati and others 2010) where *Cooperia* has been found to be the predominant species identified as persisting after IVM administration.

The results from this work suggest that IVM treatments may not be as efficacious as farmers believe with respect to removing *Cooperia*

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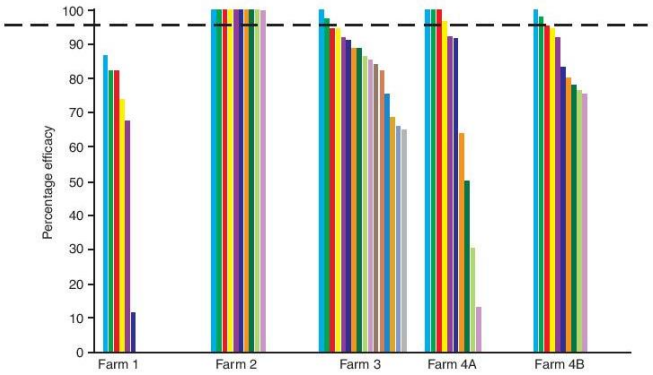

FIG 1: Percentage reduction in faecal egg counts observed in individual calves in each group after administration of injectable ivermectin. The dashed line indicates the 95% efficacy cutoff point.

TABLE 1: Data derived from the ivermectin (IVM) faecal egg count reduction tests from farms 1 to 4: faecal egg counts (FEC), percentage reduction in FEC and genus identification of 100 L_3 larvae at day 0 and 14 of IVM administration

Farm	Number of animals	Mean (sem) FEC (range) Pretreatment	Post-treatment	% Reduction (95% CI)	Genus composition Pretreatment	Post-treatment
1	6	74 (20) (16-225)	20 (5) (6-52)	72.4 (41-87)	O:15, C:85	O:4 C:96
2	10	30 (7) (9-85)	0 (0) (0-0)	100 (100-100)	O:22, C:78	
3	16	35 (4) (9-90)	5 (1) (0-19)	84.3 (77-90)	O:16, C:84	C:100
4A	10	86 (14) (2-189)	11 (3) (0-48)	87.3 (77-93)	O:38, C:62	C:100
4B	10	23 (4) (1-53)	8 (3) (0-49)	65.5 (13-86)	O:29, C:71	C:100

Because of zero FEC, post-treatment coproculture from farm 2 was not conducted.
O Ostertgia species, *C Cooperia* species

and that it is time to consider that ML AR may be a concern for the UK cattle farming industry. Although no information on animal productivity was collected here, recent reports of ML resistance have identified cattle that have been clinically affected despite receiving anthelmintics (Sargison and others 2010, Orpin 2010). In samples from these animals, *Cooperia* species was observed to comprise 65 per cent of L_3 isolated by culture one month post-treatment. Currently, the WAAVP guidelines regarding the detection of AR in cattle are based predominantly on research conducted in sheep. As cattle traditionally show lower FECs and in light of more sensitive egg counting techniques available, future recommendations need to be altered to reflect this. A recent industry-driven initiative, control of worms sustainably (COWS; EBLEX 2010), highlights practices that may reduce selection pressure for AR in nematodes in cattle and provides advice on appropriate use of anthelmintics. It is important for livestock producers, suitably qualified persons and animal health advisors to give serious consideration to the impact of intensive nematode-suppressive control programmes, although there are still opportunities to affect the outcome. Producers need to be aware of the possibility of AR particularly in *Cooperia* species and to consider the use of FECs to assist in identifying the best-practice choice of anthelmintic to administer to the animals in their care.

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Appendix 3

The results from the controlled efficacy study in Chapter 4 have been previously reported in a peer-reviewed publication: Bartley, D. J.*, McArthur, C. L.*, Devin, L. M., Sutra, J-F., Morrison, A. A., Lespine, A., Matthews, J. B. (2012) "Characterisation of macrocyclic lactone resistance in two field-derived isolates of *Cooperia oncophora*." Veterinary Parasitology 190: 454 – 460

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Characterisation of macrocyclic lactone resistance in two field-derived isolates of *Cooperia oncophora*

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ABSTRACT

The anthelmintic sensitivity of two field-derived isolates (designated FI001 and FI004) of cattle nematodes from beef farms in Scotland were investigated in a controlled efficacy test (CET). Efficacies of ivermectin pour-on (IVM-PO), IVM injectable (IVM-INJ) and moxidectin pour-on (MOX-PO) formulations were assessed. In each group, five helminth-naïve calves were infected experimentally with 50,000 third stage larvae from either isolate and administered with anthelmintic at the manufacturers' recommended dose rate 28 days later. For each isolate, nematode burdens were compared between treatment and control groups to determine efficacy. Nematode species composition, based on data derived from the untreated control groups' burden estimations, were 39 and 14% *Cooperia oncophora* and 61 and 86% *Ostertagia ostertagi* for isolates FI001 and FI004, respectively. Macrocyclic lactone resistance in *C. oncophora* was confirmed for both FI001 and FI004 isolates. Efficacies (as determined by nematode burden analysis) of 4, 21 and 31% for FI001, and 10, 1 and 74% for FI004, were obtained for IVM-INJ, IVM-PO and MOX-PO, respectively. Efficacy based on faecal egg count reduction at seven days post anthelmintic administration were 8, 99 and 100% for FI001, and 37, 20 and 100% for FI004 for IVM-INJ, IVM-PO and MOX-PO, respectively. In summary, this study details two macrocyclic lactone resistant isolates of *C. oncophora* obtained from cattle from two distinct geographical locales in the UK.

1. Introduction

Grazing cattle are susceptible to a number of gastrointestinal nematodes, with *Ostertagia ostertagi* and *Cooperia oncophora* being the most prevalent in temperate regions such as the UK (Anderson et al., 1965; Borgsteede, 1977; Claerebout et al., 1998; Rose, 1968). *O. ostertagi* is considered the more pathogenic of the two species (Bairden and Armour, 1981) and has been shown to cause profuse watery diarrhoea, inappetence and poor weight gain, with low grade infections leading to losses of around 30–60 kg in body weight gain in untreated beef cattle in their first 12 months compared to anthelmintic treated counterparts (Dimander et al., 2000, 2003). In addition to reduced weight gains, infection can reduce milk output significantly in dairy stock (Charlier et al., 2009). Although *C. oncophora* is generally considered to be a species of relatively low pathogenicity (Anderson et al., 1965; Coop et al., 1979) studies have indicated that, in co-infections with *O. ostertagi*, this parasitic nematode contributes to reduced productivity and inappetence (Hawkins, 1993; Stromberg et al., 2012; Sutherland and Leathwick, 2011).

The prophylactic use of anthelmintics is the control option most utilised by livestock producers and has been

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Table 1
Trial designs for the controlled efficacy tests, including dosage of anthelmintic, number of calves on trial per group, infective dose, days post-infection of treatment and necropsy. (P.M.)

Treatment (dosage)	Number of calves	Dose (L_3)	Day post infection	
			Treat	P.M.
Control – Untreated	5	50,000	–	35
Ivermectin injectable (0.2 mg/kg; IVM-INJ)	5	50,000	28	35
Ivermectin pour-on (0.5 mg/kg; IVM-PO)	5	50,000	28	35
Moxidectin pour-on (0.5 mg/kg; MOX-PO)	5	50,000	28	35

shown to increase productivity in both dairy (Gross et al., 1999) and beef (Dimander et al., 2000) cattle. Macrocyclic lactone (ML) anthelmintics hold the major market share for antiparasitics in cattle; partly because of their high efficacy and persistent effect against all developmental stages of parasitic nematodes of relevance, but also because of their additional efficacy against ectoparasites (Gonzalez et al., 2009). Currently, MLs are available for use in cattle as topical pour-on preparations or as a subcutaneous injection. The former method of application is most popular due to the associated reductions in animal handling time and the lower risk of injury to animals and handlers (Bogan and Armour, 1987).

Published reports of ML resistance/inefficacy in cattle nematodes have been documented in nine countries globally (Sutherland and Leathwick, 2011). In New Zealand and the US, for example, resistance to BZ and ML classes in the same nematode population have been identified (Gasbarre et al., 2009a, 2009b; Waghorn et al., 2006) and, in Argentina and Brazil, resistance of *Ostertagia*, *Cooperia*, *Haemonchus* and *Oesophagostumum* species to all three main classes of anthelmintic has been described (Anziani et al., 2004; Soutello et al., 2007). In the UK, the first reported case of ivermectin (IVM) resistant *Cooperia* nematodes in cattle was made in 1999 (Coles and Stafford, 1999; Stafford and Coles, 1999). Since then, there have been a few reports of reduced efficacy following administration of MLs in UK cattle (Orpin, 2010; Sargison et al., 2009, 2010; Stafford et al., 2010); however no extensive studies have been conducted. Previously, we reported reduced efficacy of injectable IVM against three field isolates of cattle nematodes (McArthur et al., 2011). Here, for two of these populations, we have investigated the anthelmintic sensitivity phenotype further by undertaking a controlled efficacy test (CET) in experimentally infected cattle.

2. Materials and methods

2.1. Parasite isolates

Faecal material was collected and cultured to generate infective larvae (L_3), from first season grazing calves from farms that had been identified through an ongoing anthelmintic sensitivity study (McArthur et al., 2011). In brief, L_3 were cultured from pre-treatment faecal material collected using the techniques described previously (Coop et al., 1995). Resultant L_3 were passaged through two helminth-naïve calves to produce sufficient material for use in the CET. The isolates used in this study were designated FI001 and FI004 and were derived from beef cattle farms in Dumfriesshire and Ayrshire, Scotland, respectively. On-farm IVM-faecal egg count reduction tests (FECRTs) using subcutaneous administration had demonstrated mean faecal egg count (FEC) reductions of 72% (95% confidence intervals: 41, 87) and 87% (95% confidence intervals: 77, 93) for FI001 and FI004, respectively (McArthur et al., 2011).

2.2. Controlled efficacy test

Forty helminth-free, male calves, 4–7 months-old, and housed since birth, were infected *per os* with 50,000 L_3 each (Day 0 post-infection; PI). A total of 20 calves were infected with each isolate. On day 27 PI, a FEC was performed and the calves weighed. On the basis of these parameters the calves were allocated into blocks, the blocks were then randomly assigned to a treatment group ($n = 5$ per group). Groups 1–4 and Groups 5–8 were infected with isolates FI001 and FI004, respectively. All groups were housed separately. Treatments to groups 1 and 5 were administered on Day 28 PI with IVM by subcutaneous injection (Ivomec Super®, 1% (w/v) IVM, 10% (w/v) clorsulon, Merial Animal Health, 0.2 mg/kg body weight; BW), Groups 2 and 6 were administered with pour-on IVM (Ivomec Pour-On®, 0.5% (w/v) IVM, Merial Animal Health; 0.5 mg/kg BW), Groups 3 and 7 were administered with MOX as a pour-on preparation (Cydectin Pour-On®, 0.5% (w/v) MOX, Pfizer Animal Health Ltd; 0.5 mg/kg BW). The two remaining groups (4 and 8) received no anthelmintic and acted as infection controls for the experiment (Table 1). All anthelmintic treatment doses were calculated according to the respective manufacturer's instructions, with pour-on doses rounded up to the nearest 1 ml (dosage range 0.50–0.56 mg/kg BW) and injectable doses to the nearest 0.1 ml (0.2 mg/kg BW). Pour-on treatments were applied along the midline of the back from the withers to the tailhead using a syringe, animals were observed closely for 30 min after treatment for any licking behaviour. All experimental procedures described here were approved by the Moredun Research Institute Experiments and Ethics Committee and were conducted under the legislation of a UK Home Office License (reference PPL 60/03899) in accordance with the Animals (Scientific Procedures) Act of 1986.

2.3. Samples

Faecal samples were taken *per rectum* from each calf prior to infection to confirm that they were negative for helminth eggs, on Day 27 PI prior to treatment allocation, on the day of anthelmintic treatment (Day 28) and then

daily until necropsy seven days later. Faecal egg counts (FECs) were conducted in duplicate using a modification of the technique described by Jackson (1974). Venous blood was collected via jugular venepuncture into 10 ml heparinised Vacutainer tubes (Becton Dickinson vacutainers systems) at 4, 8, 24, 48, 120, 144 and 168 h post administration of anthelmintic. All blood samples were stored in lidded cool boxes to prevent ML degradation. The samples were immediately centrifuged at 1272 × *g* for 15 min at 4 °C; plasma recovered and stored at −20 °C.

2.4. Necropsy and worm recovery

All animals were necropsied on Day 35 PI (Day 7 post treatment) using post mortem and nematode recovery methods described previously (Patterson et al., 1996). The full length of the small intestine was removed and processed to ensure that any worms that may have been temporarily paralysed and subsequently re-established to a more distal region of the gut were recovered (McKellar et al., 1988). Total nematode burdens were estimated from counts of a 2% sub-sample of the abomasal and intestinal washings and saline digests. Enumerated nematodes were classified to stage and species using criteria described in the Ministry of Agriculture, Fisheries and Food document (MAFF, 1986).

2.5. Ivermectin concentration and kinetic analysis

ML concentrations were determined in plasma by high performance liquid chromatography (HPLC) with fluorescence detection according to previously described and validated methods (Alvinerie et al., 1998). Data were analysed using a non-compartmental approach with version 4.2 of the Kinetica Tm computer program (InnaPhase, Philadelphia, USA). The partial area under the plasma concentration–time curve (AUC) from t_0 to t_{7d} was calculated by the linear trapezoidal rule. Data are expressed as mean ± standard error of the mean (SEM).

2.6. Statistical analyses

Nematode burdens and FECs were square-root transformed to successfully normalize for variance. Burdens were compared using one way ANOVA (Minitab version 13), followed by Fisher's pairwise comparisons when found to be significant ($p < 0.05$). The percentage efficacy (PE) of each treatment was calculated by means of the standard equation: $(1 - (T/C)) \times 100$ where C and T are the arithmetic mean total nematode burdens or FECs of the untreated control and treated groups, respectively (Coles et al., 1992). Anthelmintic resistance was deemed to be present when the PE in reducing nematode burden or FEC was <95%, with a lower 95% confidence limit of <90%. Bootstrap analysis was also conducted, with a re-sampling number of 2000 using the "BootStreat" program (Cabaret and Antoine, 2008) to calculate mean treatment efficacies and upper and lower 95% confidence limits. Statistical analysis for the comparison of mean AUC values was performed using one-way ANOVA followed by Fisher test (Statview software, Abacus concepts, Berkeley, CA, USA). Statistical significance was accepted as $p < 0.05$.

3. Results

3.1. Observations after treatment

One animal from the FI001 IVM-INJ group had no IVM detected in its plasma and was consequently removed from the trial and any subsequent analysis. No licking was observed in any of the animals administered with the pour-on applications over the entire observation period.

3.2. Nematode burden analysis

The average percentage establishment of nematodes in the control calves was 27% (13,630 nematodes) and 35% (17,560 nematodes) for FI001 and FI004, respectively. In terms of species composition identified at post mortem in these groups, FI001 comprised 61% *O. ostertagi* and 39% *C. oncophora* and FI004 comprised 86% *O. ostertagi* and 14% *C. oncophora*. In terms of efficacy, when compared to the untreated control group, for FI001, mean reductions in *C. oncophora* were 38%, 64% and 31% in the IVM-INJ, IVM-PO and MOX-PO treatment groups, respectively (Table 2). For FI004, the observed mean reductions in this nematode species were 10%, 0% and 74% for the IVM-INJ, IVM-PO and MOX-PO treatment groups, respectively. All three anthelmintic treatments produced a mean reduction in *O. ostertagi* of >99.5% in both isolates. For isolate FI001, no significant differences in nematode burdens were observed when the anthelmintic treated groups were compared with the non-treated control group, whereas with isolate FI004, significant reductions in nematode burdens were only observed in the group administered with MOX-PO ($p < 0.05$).

3.3. Faecal egg count analysis

Fig. 1A and B shows the mean FECs obtained daily for each group from 0 to 7 days after anthelmintic administration. FECs in the untreated control groups were consistently high throughout the sampling frame, with mean FECs across the 7 days of 1245 and 674 EPG for isolates FI001 and FI004, respectively. In animals that received isolate FI001, FECs declined steadily from 0 to 4 days after administration of IVM-PO and from 0 to 5 days after administration of IVM-INJ and MOX-PO. The pattern of faecal egg output after anthelmintic administration differed with isolate FI004, as only in those animals that received MOX-PO did the FECs reach 0 EPG (at four days after treatment). In the groups that received IVM, although the FECs declined after treatment, they remained >0 EPG throughout. For isolate FI001, at seven days after treatment, the mean FECR observed was 98%, 99% and 100% in the groups that received IVM-INJ, IVM-PO and MOX-PO, respectively. In contrast, for isolate FI004, mean FECRs of 37%, 20% and 100% were observed at seven days after treatment with IVM-INJ, IVM-PO and MOX-PO, respectively (Table 3). Significant differences ($p < 0.05$) in FEC compared to non-treated control animals were observed with all treatment groups with isolate FI001,

Table 2
Arithmetic mean (± S.E.M.) small intestine worm counts, range of counts, differentiation of worm burdens into male, female and juvenile worms, and percentage efficacy (P.E.) of anthelmintic treated groups of calves relative to untreated control calves seven days post-treatment.

Treatment	Arithmetic mean worm burden (± S.E.M.) [range]				Percentage efficacy
	Male	Female	Juvenile	Total	
Isolate FI001					
Untreated	2480 (±635) [400–3800]	2810 (±633) [900–4400]	60 (±29) [0–150]	5350 (±1288) [1300–8350]	NA
Injectable ivermectin (IVM-INJ)	1425 (±429) [400–2600]	1900 (±625) [200–3450]	0 (±0) [0–0]	3325 (±1051) [600–6050]	38
Pour-on ivermectin (IVM-PO)	920 (±543) [50–3000]	1010 (±557) [0–3050]	0 (±0) [0–0]	1930 (±1098) [50–6050]	64
Pour-on moxidectin (MOX-PO)	1590 (±837) [0–3700]	2090 (±983) [0–5000]	20 (±20) [0–100]	3700 (±292) [0–8550]	31
Isolate FI004					
Untreated	1170 (±326) [250–2050]	1270 (±339) [350–2050]	0 (±0) [0–0]	2440 (±679)[a] [850–3900]	NA
Injectable ivermectin (IVM-INJ)	1040 (±97) [850–1400]	1160 (±73) [1000–1350]	0 (±0) [0–0]	2200 (±104) [a] [1900–2400]	10
Pour-on ivermectin (IVM-PO)	1280 (±270) [750–2300]	1430 (±167) [1100–1950]	0 (±0) [0–0]	2710 (±428) [a] [1850–4250]	0
Pour-on moxidectin (MOX-PO)	350 (±183) [0–1000]	290 (±97) [100–650]	0 (±0) [0–0]	640 (±274) [b] [100–1650]	74

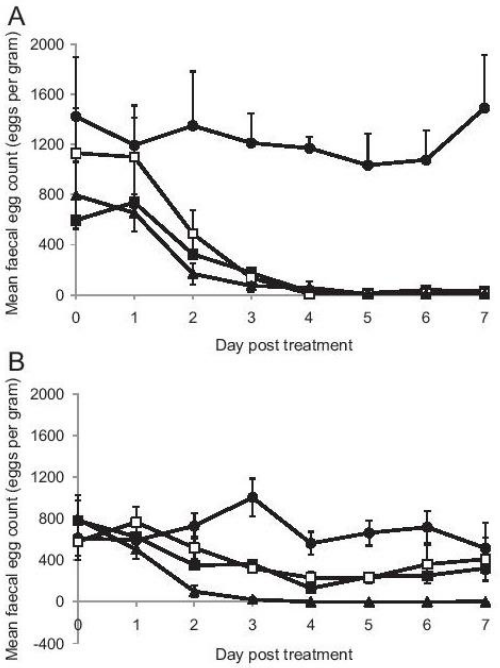

Fig. 1. Arithmetic mean faecal egg counts (±standard error of the mean, SEM) of the four groups of calves 28–35 days post infection with 50,000 infective larvae; FI001 (A) and FI004 (B). The groups are untreated control (●); injectable ivermectin (■); pour-on ivermectin (□) and pour-on moxidectin (▲).

whereas with isolate FI004, significant reductions in FEC were only observed with the group administered with MOX-PO.

3.4. Analysis of IVM and MOX concentration kinetics in plasma

Fig. 2A and B shows the mean plasma concentrations of IVM or MOX measured over time for each treatment group for both isolates and area under plasma concentration–time curves. In the IVM-INJ groups, the mean peak IVM concentration was between 20.4 and 38.6 ng/ml. In the IVM-PO groups, the mean peak IVM concentration was between 24.5 and 26 ng/ml, whereas the mean peak MOX concentration in MOX-PO treatment groups was 7.9 and 12.4 ng/ml.

4. Discussion

The current study demonstrated a lack of ML (IVM and MOX) efficacy against *C. oncophora* present in nematode isolates derived from two UK beef cattle enterprises. To date, reports of resistance/inefficacy in the UK have only described experiments in which pour-on IVM (Coles et al., 2001; Sargison et al., 2010) or doramectin (Sargison et al., 2009) were administered and these reports were

Table 3
Arithmetic mean (± S.E.M) [range] faecal egg counts and percentage efficacy (P.E.) of anthelmintic treated groups of calves relative to untreated control calves seven days post-treatment.

Treatment	Faecal egg count (eggs per gram)		Percentage efficacy (95% CI)	
	Day 0	Day 7	WAAVP	Bootstrap estimate
Isolate FI001				
Untreated	1425 (±472) [446–2624]	1491 (±421) [675–2741]	NA	NA
Injectable ivermectin (IVM-INJ)	596 (±91) [486–869]	30 (±9) [4–44]	98 (95, 99)	98 (95, 100)
Pour-on ivermectin (IVM-PO)	1132 (±357) [491–2453]	13 (±11) [2–57]	99 (95, 100)	99 (97, 100)
Pour-on moxidectin (MOX-PO)	795 (±268) [252–1715]	4 (±2) [0–12]	100 (99, 100)	100 (99, 100)
Isolate FI004				
Untreated	613 (±165) [189–1184]	515 (±249) [126–1458]	NA	NA
Injectable ivermectin (IVM-INJ)	781 (±243) [365–1562]	323 (±41) [221–428]	37 (0, 77)	22 (0, 69)
Pour-on ivermectin (IVM-PO)	578 (±172) [116–972]	411 (±208) [113–1215]	20 (0, 81)	0 (0, 77)
Pour-on moxidectin (MOX-PO)	798 (±178) [347–1305]	1 (±0) [3–0]	100 (99, 100)	100 (99, 100)

based on FEC reduction alone. Although there have been no reports of MOX resistance in cattle nematodes in the UK, there has been data describing this phenomenon in cattle from Argentina (Anziani et al., 2001), Belgium (El-Abdellati et al., 2010), Brazil (Condi et al., 2009), New Zealand (Vermunt et al., 1996) and the US (Gasbarre et al., 2009a, 2009b). As in the case of ovine nematodes (Pomroy and Whelan, 1993), reports of side resistance between members of the ML class in bovine nematodes are not unexpected (Conder et al., 1993; Vermunt et al., 1996). Here, for isolate FI004, the mean efficacy of MOX-PO was greater than IVM-PO and IVM-INJ, as assessed by nematode burden analysis, although this was not the case with isolate FI001. In previous reports of ML resistance in cattle, where both compounds were tested, MOX treatment generally resulted in higher percentage reductions in nematode burden and FEC than IVM (Anziani et al., 2001; El-Abdellati et al., 2010; Gasbarre et al., 2009b) although this was not always the case (Vermunt et al., 1996). The higher efficacy of MOX is believed to be due the compound's greater lipophilicity, as well as higher potency and persistency when compared to avermectins such as IVM (Kieran, 1994).

The drug concentration data indicates adequate uptake of the anthelmintics administered and confirms that the nematodes are ML resistant. Macrocyclic lactones have systemic action and their concentration and persistence in plasma and in tissues where parasites are located, contribute to their efficacy against the target parasite species. In this study, the ML concentration profiles in plasma were within the limits expected in cattle for each of the treatment groups (Gayrard et al., 1999; Lifschitz et al., 1999a, 1999b; Sallovitz et al., 2002). Generally, subcutaneous

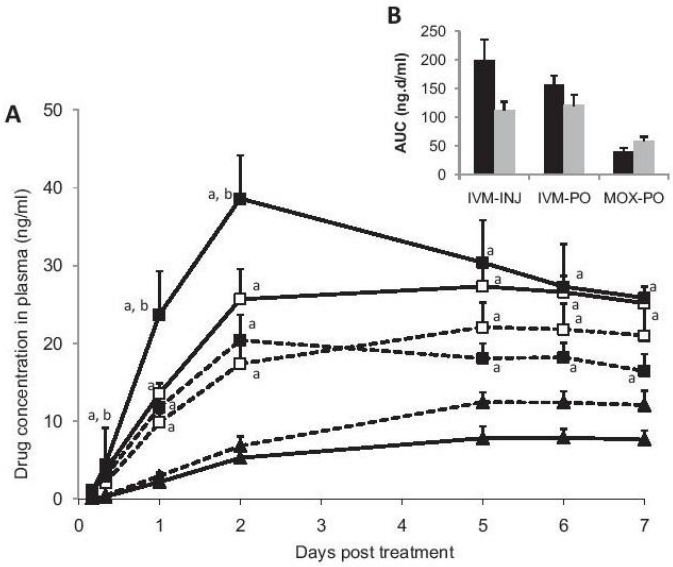

Fig. 2. (A) Plasma profile of macrocyclic lactone concentration in field derived nematode infected cattle: isolate FI001 (full line) and isolate FI004 (dotted lines) treated at time 0. IVM-INJ (■), IVM-PO (□) and MOX-PO (▲). (B) Area under plasma concentration–time curve (AUC): isolate FI001 (■) and isolate FI004 (▩). Values are arithmetic means ± S.E.M. Values are significantly different when compared with MOX-PO (a) or when compared with IVM-PO (b).

injection is the most efficient route for ML administration in terms of bioavailability in cattle and other species, when compared to oral and topical administration (Gayrard et al., 1999; Laffont et al., 2001; Lespine et al., 2005). Ivermectin and MOX have different plasma and tissue kinetics which affect the duration of activity and generally, MOX has a longer persistence of activity than IVM does in hosts infected with ML sensitive nematodes.

The principal species surviving ML treatment in both isolates was *C. oncophora*. This is consistent with previous findings in the EU, where this species predominated after IVM treatment (Demeler et al., 2009; El-Abdellati et al., 2010; Familton et al., 2001). *C. oncophora* was also demonstrated to dominate larval cultures derived from samples obtained after IVM treatment failure in the field in Scotland (Sargison et al., 2009). *Cooperia*, including *C. oncophora*, are known to be one of the dose-limiting species for IVM (Benz and Ernst, 1979; Bisset et al., 1990; Egerton et al., 1979; McKenna, 1995), with similar findings reported for MOX (Ranjan et al., 1992; Vermunt et al., 1996; Whang et al., 1994).

Cooperia species have often been cited as relatively non-pathogenic (Anderson et al., 1965; Coop et al., 1979); however, ill thrift has been reported in cattle harbouring suspected or confirmed anthelmintic resistant nematodes of this species (Anziani et al., 2001; McKenna, 1995; Sargison et al., 2010). Indeed, recent studies in New Zealand have indicated that failure to control this nematode species in yearlings results in a 14 kg difference in live-weight gain over a grazing season compared to uninfected control animals (Sutherland and Leathwick, 2011). Previous studies in Scotland also indicated that this was the case (Armour et al., 1987). Some authors have suggested that anthelmintic resistant *C. oncophora* are more pathogenic than ML sensitive worms of the same species (Coles et al., 2001; Njue and Prichard, 2004). The trial here was of too short a duration to assess the clinical effects of the parasite isolates investigated and further studies need to be performed to explore this further.

Lower FECs were observed in calves infected with FI004 compared to those infected with FI001. This difference may be attributable to higher proportions of *O. ostertagi* in the FI004 isolate. *O. ostertagi* is known to be less fecund than *Cooperia* species (Kloosterman, 1971).

Examination of the FEC data following anthelmintic administration demonstrated that there was suppression of egg production from worms that survived treatment. It is believed that although nematodes are able to survive in therapeutic concentrations of the compound, IVM is still able to paralyse the uterine musculature (McKenna, 1997; Scott et al., 1991) resulting in a suppression in egg output in surviving worms. Egg output may then resume as the local anthelmintic concentrations fall over time, although one report detailed the effect of IVM on the reproductive potential of *Cooperia curticei*, with reduced numbers of eggs observed in worms from IVM treated animals compared to worms in untreated controls (McKellar et al., 1988). Although assessed at 7 days after anthelmintic administration, all treatments for isolate FI001 gave FECR efficacies >95%, whereas for isolate FI004, only MOX-PO treatment resulted in a FECR >95%. Whilst the faecal egg counts of the treated groups may have increased if left until 14 days after anthelmintic administration, as per the current WAAVP guidelines (Coles et al., 2006), it highlights the limitation of examining FECs in isolation when assessing anthelmintic efficacy.

The data generated here and in other trials reaffirms the need to ensure that appropriate guidelines are followed when assessing anthelmintic efficacy (Coles et al., 2006).

Competing interest

The authors declare that they have no competing interests.

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